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EMERGING INFECTIOUS DISEASES PROGRAM
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Abstract

Title of Dissertation:

The Fate of the Red Cells: Insights from two Models of Severe Malarial Anemia

Juliana Valentina Harris, Doctor of Philosophy, 2011

Thesis directed by:

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Malaria, a disease caused by an intracellular parasite of the genus *Plasmodium*, causes 350-500 million cases annually with 1-2 million deaths. The majority of these deaths occur in children in sub-Saharan Africa due to complications of *P. falciparum* infection, such as severe malarial anemia (SMA). The pathogenesis of SMA is complex and not well understood; however, it is known that there is greater destruction of red cells than can be accounted for by the parasite alone. The question of what is happening to the uninfected red cells remains unanswered. We therefore investigated the pathogenesis of SMA by studying the fate of uninfected red cells in two different mouse models. Based on findings in patients with SMA showing an acquired deficiency in complement regulatory proteins and increased susceptibility to complement, we emphasized investigating the role of complement and complement regulatory proteins in the development of SMA.

The first model we utilized was a well established model of malarial anemia involving the rodent parasite *P. chabaudi* in C57BL/6 mice. We ascertained that Crry partial deficiency did not result in more severe anemia evident by blood counts, but did result in a higher level of erythrophagocytosis which could be negated with supplementation of the deficient protein. These findings indicate that Crry is important in red cell protection during a malaria infection. Since Crry, whose function is to protect host cells from complement attack, is important in red cell protection during a malaria infection, does that necessarily mean C3 is deleterious and/or causes more severe anemia? Our results in C3 deficient animals suggest that it may be harmful since C3 deficient animals had less severe anemia; however, these animals still developed anemia and were able to clear and sequester transferred red cells.

In order to study SMA in a model more representative of *P. falciparum* infection, we developed the second model that was used to investigate SMA pathogenesis. We showed that *P. berghei* infection following recovery from *P. chabaudi* infection in C57BL/6 animals resulted in anemia with a low level parasitemia. Characterization of the model revealed similarities to anemia in *P. chabaudi* infected animals, such as increased organ pathology and erythrophagocytosis. This model can now be used to evaluate factors such as complement and complement regulatory proteins in the development of anemia.

The Fate of the Red Cells: Insights from two Models of Severe Malarial Anemia

By

Juliana Valentina Harris

Dissertation submitted to the Faculty of the Emerging Infectious Diseases Interdisciplinary Graduate Program of the Uniformed Services University of the Health Sciences F. Edward Hébert School of Medicine in partial fulfillment of the requirements for the degree of Doctor of Philosophy 2011

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Dedication

To my Mom who has stood by me through all the ups and downs, always had words of encouragement when the going got tough, and never stopped believing we would see this day. Thank you for indulging a little girl with her first chemistry set and encouraging me to follow my dreams!

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Chapter 1

Introduction

Malaria

Organism and Significance

Malaria has been plaguing mankind for centuries. There are references to a seasonal and intermittent fever in ancient Chinese, Indian, and Assyrian medical and religious texts. Hippoccrates, in the 5th century BC, was the first person to describe in detail the clinical picture of malaria and complications of the disease[1]. However, it wasn't until 1880 that malaria parasites were first seen and described in human blood by Charles Laveran and an understanding of how malaria parasites were transmitted did not occur until the late 1890s[1].

Malaria is a mosquito-borne disease caused by an intracellular parasite of the phylum Apicomplexa and the genus *Plasmodium*. Four species of *Plasmodia* (*P. falciparum, P. vivax, P. ovale,* and *P. malariae*) are responsible for causing disease in humans. *P. falciparum* and *P. malariae* are found worldwide, although the prevalence of *P. malariae* is low. *P. vivax* is found primarily in Asia, Latin America, and to a lesser extent Africa, while, *P. ovale* is found mostly in Africa and occasionally the islands of the West Pacific. Of the four human malarias, *P. vivax* and *P. falciparum* are the most abundant and *P. falciparum* causes the most severe disease.[2]

While malaria is distributed primarily in the tropics and is considered a disease of the developing world, it is still of interest to developed countries because of the global impact of malaria. Over 3 billion people live in at risk areas and approximately 1 million

deaths annually are attributed to malaria[3]. While malaria is no longer natively found in the United States, we are interested in malaria because of the risk to travelers and military personnel. Malaria has essentially always had an effect on our military personnel/operations. There were over 113,000 cases, 3.3 million sick days, and 90 deaths due to malaria in American troops in World War II[4]. Nowadays, due to increasing drug resistance and lack of a vaccine, our military personnel are at risk again for infection and the development of severe disease because of lack of any sort of immunity. In addition to military and traveler risk, malaria is a huge economic burden. The cost for supporting the minimal set of interventions to effectively control malaria is estimated at $3.2 billion per year, with $1.9 billion needed just for Africa[3]. Because of the global impact of malaria, several global initiatives have been developed with the goal of eradiating malaria.

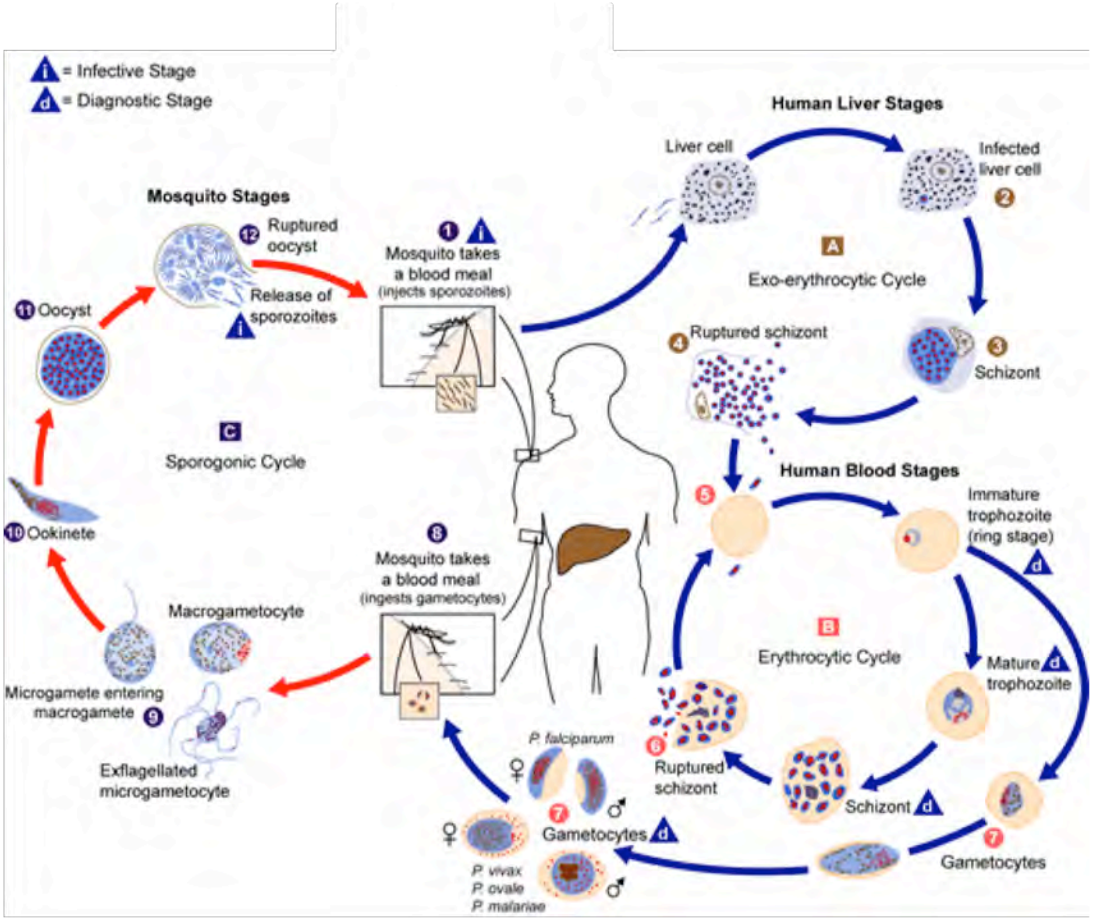
Life cycle

As with many other parasites, the *Plasmodium* life cycle is complex and requires multiple hosts. The life cycle alternates between sexual reproduction in the definitive female *Anopholes* mosquito host and asexual reproduction in the intermediate mammalian host[5]. The asexual reproductive phase of the life cycle is further divided into two parts, the exo-erythrocytic and erythrocytic cycles[5]. It is the erythrocytic cycle that causes symptomatic disease.

Figure 1 depicts the full life cycle of the *Plasmodium* parasite where a human is the intermediate host. A malaria-infected female *Anopheles* mosquito taking a blood

Figure 1: Life cycle of the malaria parasite. A malaria-infected female *Anopheles* mosquito takes a blood meal and inoculates the human host with sporozoites. Sporozoites then infect liver cells and mature into schizonts (exo-erythrocytic cycle), which rupture and release merozoites that then infect red blood cells. Within the red cell the parasite undergoes asexual multiplication, ultimately rupturing and releasing merozoites that can infect more red blood cells (erythrocytic cycle). Parasites in the blood stage are responsible for clinical disease. Occasionally a parasite in a red cell will mature into a microgametocyte (male) or macrogametocyte (female) that can then be ingested by a female *Anopheles* mosquito during a blood meal. Within the mosquito gut the micro- and macrogametocytes fuse together and undergo several maturation stages ultimately resulting in an oocyst outside the midgut that ruptures and releases sporozoites. Sporozoites migrate to the mosquito salivary glands and upon taking another blood meal the infected mosquito will inoculate a new human host, thus continuing the cycle.

Figure 1: Life cycle of malaria parasites



(http://www.dpd.cdc.gov/dpdx/HTML/Malaria.htm)

meal will inoculate the human host with its saliva containing the infectious sporozoites. The sporozoites leave the blood and invade hepatocytes[6] where the exo-erythrocytic cycle occurs. Within the hepatocyte the parasite matures in 5-16 days (length of time depends upon the species of parasite) into a schizont containing 30,000-50,000 merozoites[1]. In some species, *P. vivax* and *P. ovale*, following hepatocyte invasion, some of the parasites enter a dormant phase called hypnozoite where development is ceased for many days/weeks until an unknown stimulus triggers schizogony to continue. This dormancy allows for relapsing infections. Upon schizont and subsequent hepatocyte rupture, the merozoites are released into the liver sinusoids, where they escape and within approximately 15 minutes invade erythrocytes to initiate the erythrocytic cycle. Within the erythrocyte, the merozoite undergoes asexual multiplication by maturing from a ring form to a trophozoite into a schizont containing 8-24 merozoites. The infected erythrocyte with a mature schizont ruptures releasing the merozoites, which can then invade additional erythrocytes. The duration of erythrocytic cycles differs between malaria species, lasting 48-50 hours for *P. falciparum, P. vivax,* and *P. ovale* and 72 hours for *P. malariae*. It is the rupture of erythrocytes and subsequent release of toxic debris that causes symptomatic disease.

Most merozoites invading erythrocytes will undergo asexual schizogony; however, a small proportion of the merozoites will develop into the first sexual stage, gametocytes. When a female *Anopheles* mosquito takes a blood meal from an infected human, she ingests the male and female forms of the gametocyte (microgametocyte and macrogametocyte respectively). In the mosquito gut, the gametocytes escape the erythrocyte, microgametocytes undergo exflagellation, and the male and female gametes

fuse to form a zygote. The zygote transforms into an ookinete and invades the mid-gut where it differentiates into an oocyst, which grows and releases sporozoites. Sporozoites then migrate to the mosquito salivary gland where they can be inoculated into a new human host when the mosquito takes another blood meal, thus perpetuating the life cycle.

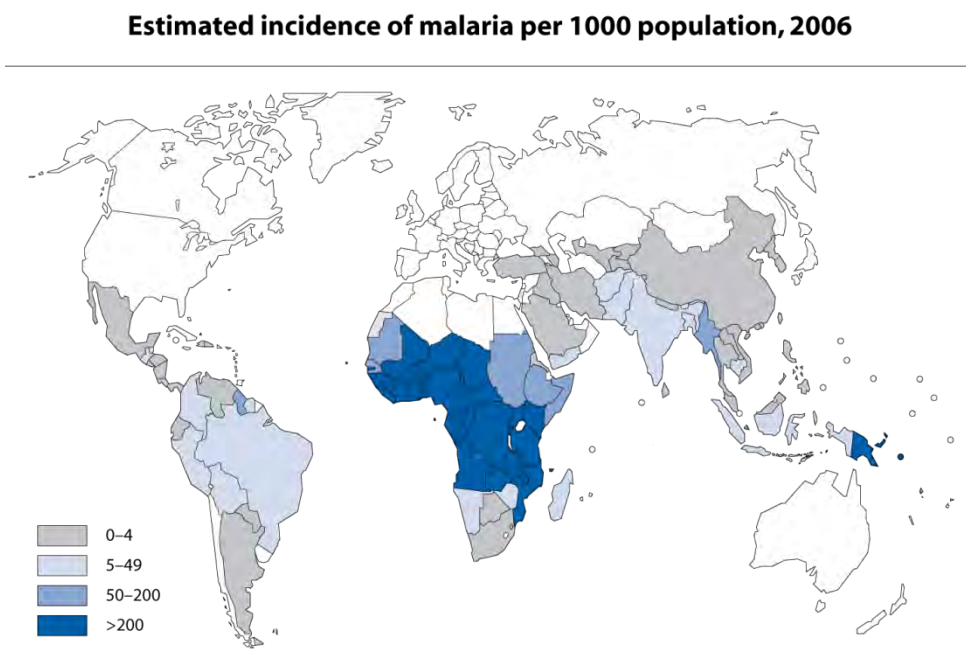
Epidemiology

Malaria is found worldwide; however, it is considered primarily a disease of the developing world. There are currently 3.2 billion people living in at risk areas for malarial transmission[3]. It is estimated that 350-500 million cases, mostly *P. falciparum* and *P. vivax*, occur annually with more than 1 million deaths[3]. The worldwide concerted effort to decrease the burden of malaria appears to be successful, as the World Health Organization (WHO) estimates there were 225 million cases of malaria with 781,000 deaths worldwide in 2009[7]. Of those deaths, 91% occurred in Africa and 85% occurred in children less than 5 years of age[7]. The greatest burden of disease is in Africa, particularly sub-Saharan Africa where approximately 60% of the worldwide malaria cases and 75% of the worldwide *P. falciparum* cases occur[3]. Of the cases that occur in this region, approximately 18% of deaths occur in children less than 5 years of age[3].

Human malaria parasites are found worldwide, primarily in the tropics and subtropics (Figure 2). *P. falciparum* is one of the most abundant and virulent species, and is found primarily in sub-Saharan Africa, Southeast Asia and the Pacific (Figure 2). It can occasionally be found in South America as well. *P. vivax* is also abundant and is found primarily in the Americas and Asia. *P. malariae* and *P. ovale*, found worldwide

Figure 2: Worldwide distribution of malaria. Malaria parasites are found worldwide, with the greatest incidence of infection in the tropics and subtropics. Malaria transmission occurs in areas of Central and South America, Africa, South and Southeast Asia. *P. falciparum* cases, while distributed worldwide, have the highest focal location in Africa.

Figure 2: Worldwide distribution of malaria



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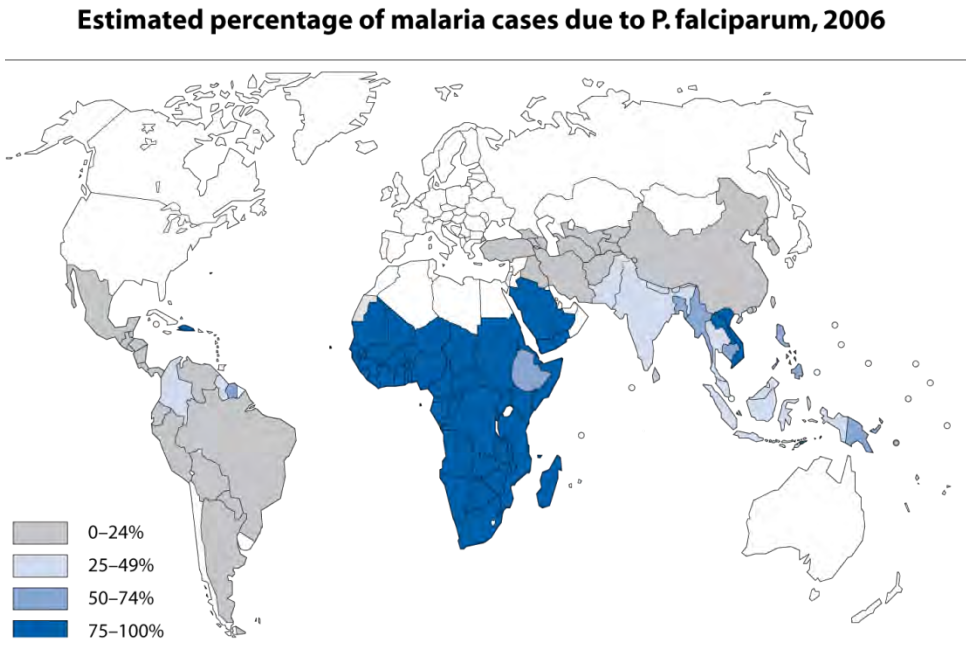


(http://gamapserver.who.int/mapLibrary/Files/Maps/Malaria_Cases_Pfalciparum_2006.png)

and mostly in Africa respectively, are only responsible for a small portion of worldwide malaria cases.[2]

Understanding the epidemiology of malarial disease requires an understanding of the patterns of transmission, which vary in regions depending upon ecological conditions and variations between the parasite and *Anopheles* vector[3]. Malaria endemicity can be classified into four groups: hypo-, meso-, hyper-, and holoendemicity[1]. Hypoendemic areas have little transmission, mesoendemic areas have intermediate transmission, holoendemic areas have transmission throughout the year and immunity outside of childhood is seen, and hyperendemic areas have intense seasonal transmission with insufficient immunity[1]. An example of how endemicity affects disease epidemiology is seen with two types of severe malaria, cerebral malaria (CM) and severe malarial anemia (SMA). CM is typically seen in areas of low endemicity, with seasonal transmission, while SMA is more often found in regions with high endemicity[8].

Diagnosis and Disease

While severe disease is a serious problem and a major cause of morbidity and mortality, malaria infections are generally curable if diagnosed and treated promptly. Diagnosis of malaria is generally performed with a blood smear or by rapid diagnostic tests. The WHO recommends artemisinin-based combination therapy (ACT) for treatment of uncomplicated malaria and IV artesunate for treatment of severe malaria[9]. Additionally, treating severe malaria involves supportive care and management of symptoms. Unfortunately, due to the complex life cycle of malaria and lack of long term immunity there has been no successful vaccines developed; however, there is a large

focus of research directed at vaccine development. Successful eradication of malaria, which is the ultimate goal, will have to involve combating the disease on 2 fronts: 1) the vector side by vector control and use of insecticide treated bednets to limit exposure and 2) the host side by gaining a better understanding of host-parasite interactions to effectively treat/cure and prevent disease.

Malaria is a febrile illness that can range in severity of symptoms from asymptomatic to mild/uncomplicated to severe/complicated and death. The clinical symptoms associated with malaria are all caused by the blood stage parasite and correspond with the rupture of schizonts, where parasite waste products and other toxic materials are deposited into the bloodstream. Classical malaria fever is characterized by the febrile paroxysm, which consists of 3 stages: the cold stage (15-60 minutes), where a patient suddenly feels inappropriately cold and shivers; the hot stage (2-6 hours), where patients stop feeling cold and become unbearably hot with temperatures that can peak at 104-106 °F; and the sweating stage (2-4 hours), where the fever breaks and symptoms diminish[1]. The time between paroxysms depends upon the species of parasite a person is infected with; typically every 48 hours for *P. falciparum, P. vivax,* and *P. ovale* and every 72 hours for *P. malariae*. In reality the classical paroxysm is rarely observed, rather patients tend to present with flu-like symptoms, which can be accompanied by other findings such as very high temperatures, enlarged liver and spleen, and increased respiratory rates.

In a small percent of cases, mostly with *P. falciparum* infection, complications occur that result in severe disease manifestations. Severe manifestations are usually a result of insufficient treatment in the early uncomplicated phase of infection[9]. In Africa,

the two most common complications are cerebral malaria and severe anemia[10;11]. Cerebral malaria (CM) is a life-threatening complication involving neurologic abnormalities, including seizures and coma. Sequestration of mature parasites in the microvasculature of the brain is thought to have a major role in the pathogenesis of CM[12]. Much research has been conducted regarding cerebral malaria and more information can be found in several review articles[13-16]. Severe malarial anemia (SMA), which is the focus of my research, is described in more detail below.

Severe Malarial Anemia

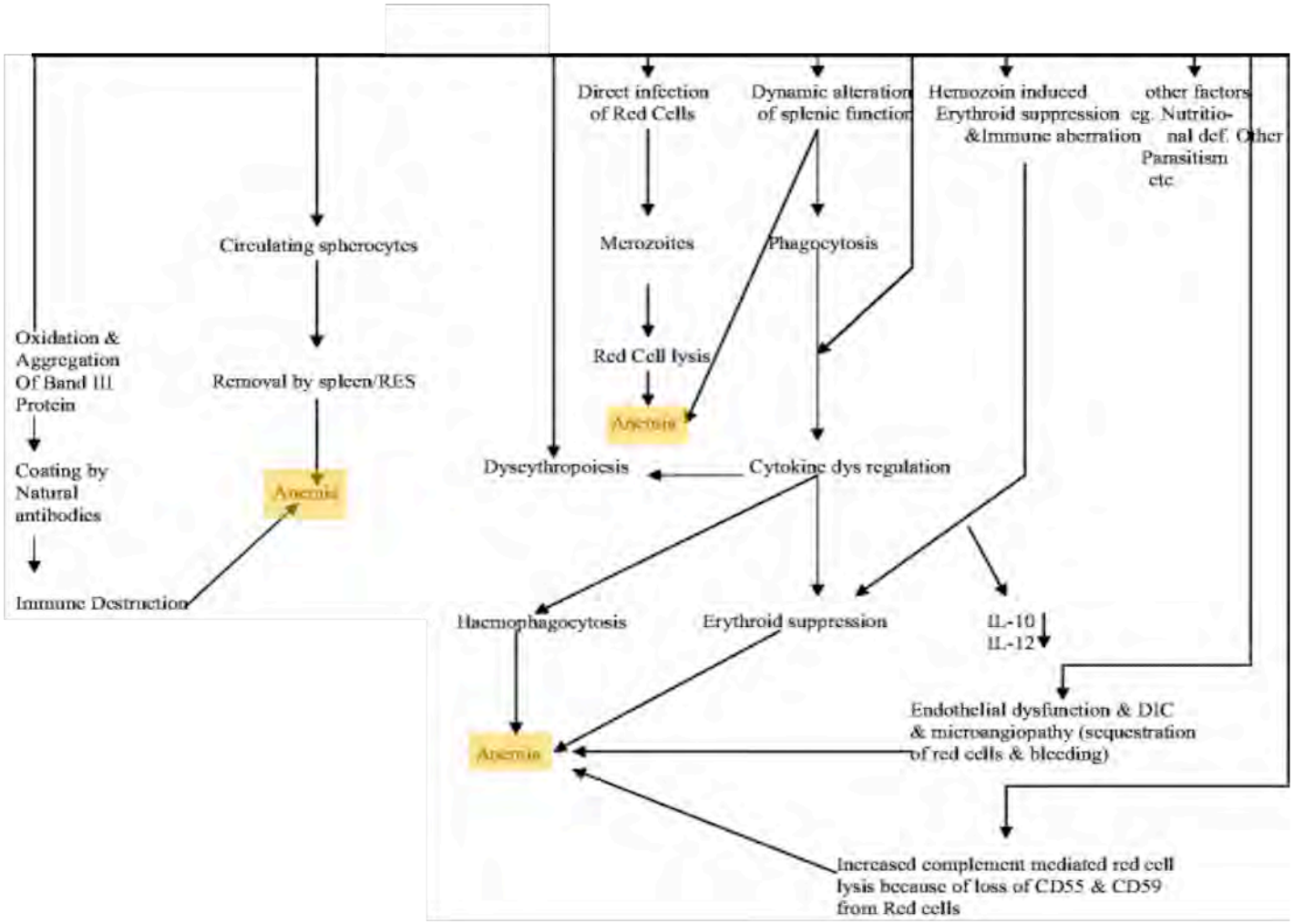
SMA is a complication of *P. falciparum* infection and is a major cause of morbidity and mortality in malarial infections. SMA is most often found in areas of high malaria transmission, where children and pregnant women are at the highest risk[8]. SMA is the leading cause of death in children with malaria and the mean age of cases is 1.8 years[11]. The WHO defines SMA as a hemoglobin concentration less than 5 g/dL or a hematocrit < 15% with a parasitemia of >10,000 parasites/μL[11]. However, this strict definition is not very universal as there are multiple factors in different regions that affect "normal" parameters. Additionally, patients in some areas have had severe anemia with no apparent parasitemia, but have responded positively to antimalarials[17].

Regardless of how SMA is defined, the pathogenesis of the disease is not well understood. There are multiple causes of anemia in a malaria infection[18] (Figure 3) and it is likely that several of the mechanisms are involved in the pathogenesis of SMA. Several reviews[18-21] have been written regarding SMA, which concur with the idea that the pathogenesis of SMA is multifactorial. Anemia during a malaria infection can be

attributed to three factors: destruction of infected erythrocytes, destruction of uninfected erythrocytes, and ineffective erythropoiesis. Infected erythrocytes are lysed as a direct result of the parasite life cycle; however, the amount of red cell destruction that occurs by this means cannot account for the amount of red cell destruction in SMA[22;23]. Many studies, in humans and animals, have examined the erythropoietic response during a malaria infection. Erythropoietic suppression and dyserthropoiesis have been implicated as a mechasnism involved in the development of SMA[24-30]. Additionally, destruction of uninfected red cells is important in the development of SMA. The means by which the RBCs are being destroyed is not clear. Patients with SMA have been shown to have an acquired deficiency in complement regulatory proteins and/or increased susceptibility to complement[31-35]. The increased deposition of C3 on erythrocyte surfaces and the increased immune complexes could result in clearance by phagocytes; erythrophagocytosis has been observed in humans and mice with malaria[36;37]. Additionally, studies have shown binding of *Plasmodium* rhoptry proteins to the surface of uninfected red cells and antibodies targeted against those proteins has been shown to induce red cell destruction by phagocytosis and complement activation[38;39].

Figure 3: Causes of anemia in a malaria infection. There are several mechanisms that can lead to anemia during a malaria infection. Infected red cells undergo lysis as a result of the parasite life cycle. Various immune-mediated events, from both the innate and adaptive arm of the immune system, result in enhanced clearance and destruction of infected and uninfected red cells. Finally, ineffective erythropoiesis from bone marrow suppression results in the inability to replace lost red cells.

Figure 3: Causes of anemia in a malaria infection



Modified from image in Ghosh, K. (2007)[18]

Immune Response to Malaria

Given the complexity of the malaria life cycle, it is not surprising the immune response to the parasite is equally complex. *Plasmodium* parasites express different antigens with each life stage, which necessitates cellular and/or humoral immune responses that target those specific stages in addition to more broad reacting innate responses. Additionally, the majority of parasite antigens are polymorphic and populations of parasite can exhibit antigenic variation within an individual host[1], which adds to the difficulty in developing a protective immune response. Parasite proteins found on the surface of the *Plasmodium* parasite, such as circumsporozoite protein (CSP) and merozoite surface proteins (MSPs), and found on the surface of red cells, such as parasite erythrocyte membrane protein 1 (*Pf*EMP-1) and ring-infected erythrocyte surface antigen (RESA), have been found to be immunodominant but are also polymorphic and antigenically variable[40-42].

With the different life stages and antigen variation, what effecter mechanisms are employed in an attempt to protect the host during a malaria infection? The innate and adaptive arms of the immune system are critical for control of a malaria infection. Innate responses are triggered at some unknown threshold of parasitemia and are important in controlling an exponential rise in parasite levels; however, adaptive immunity is required for elimination of the parasite[43;44]. Innate responses rely upon activation of phagocytic cells, which have been shown to release toxic oxygen species and nitric oxide and its derivatives[45-47]. Complement could also be important in the initial response by opsonization of the parasite and infected red cells for clearance by phagocytosis. Additionally, early cytokine (primarily interferon-γ (IFN-γ) and tumor necrosis factor

(TNF)) production by NK and γδT cells has been shown to occur[48-51]; although there is debate whether these responses are ultimately protective or lead to immunopathology.

In addition to innate responses, the adaptive immune response is important in protection during a malaria infection. Doolan *et al*[52] wrote a comprehensive review regarding acquired immunity. The exact role of cell-mediated immunity (CMI) in malaria is unclear, particularly because the parasite resides primarily in red cells, which do not express HLA. However, evidence does suggest that T cells, particularly $CD8^+$, are important in killing parasitized hepatocytes[53;54]. Additionally, the production of cytokines such as IFN-γ and TNF by T cells and IL-12 by antigen presenting cells appears to be important in the cell-mediated response to infection. A balance of cytokine production however seems to be necessary because elevated levels of some cytokines have been linked to more severe forms of disease[48;55;56]. While the role of CMI is not clear, humoral immune responses are clearly implicated in the immune response to malaria. Protective antibodies inhibit parasite expansion, but require the cooperation of macrophages[57]. Clearance of parasitized cells is achieved in part by enhanced splenic clearance due to Fc-mediated phagocytosis[58;59]. Additionally, passive transfer of serum from hyperimmune individuals has been shown to be temporarily protective[60-62]. The development of protective immunity to malaria depends upon constant exposure to the parasite. Sterile immunity does not develop, but rather an acquired immune response that is capable of limiting parasite density and protecting against clinical manifestations[43;63].

Understanding the immune response to infection is important for the development of an effective vaccine; however, malaria vaccine development seems to have provided more insight into the immune response towards malaria. The administration of irradiated

sporozoites has long been known to produce protection against liver stage parasites[64;65], in particular by antibody blocking invasion/maturation within hepatocytes. In particular, antibodies directed against CSP were found to be protective and subsequent cloning of CSP and development of the RTS,S/AS vaccine, which has known B and T cell epitopes, was produced[66]. Many vaccine studies have demonstrated an important role for antibodies; however, more recent work is beginning to elucidate a role for cellular immunity, particularly a role for T cells. Whole cell vaccines utilizing an ultra-low dose nonattenuated parasitized red cell[67;68] have been shown to induce a protective and robust T cell response[69]. Vaccine studies indicate a role for both cellular and humoral immunity in protection from malaria, yet these responses are not clearly apparent in natural infections. However, a better understanding of immune mechanisms seen in the vaccine studies can provide suggestions as to potential mechanisms that are in fact occurring in malaria infected people.

Complement

The Complement System

The complement system is a humoral component of the innate immune system, comprised of more than 30 serum and membrane bound proteins. As reviewed by Walport[70;71], complement has 3 main activities: 1) defending against infection by opsonization for phagocytosis, attraction of leukocytes (primarily phagocytes) to the site of activation, and direct lysis; 2) linking innate and adaptive immunity by enhancing immunologic memory and augmenting antibody responses; and 3) clearance of waste products from inflammation and immune complexes. Activation of the complement

cascade can occur by 3 pathways: classical (antigen:antibody mediated), alternative (spontaneous hydrolysis of C3), and lectin (mannose-binding lectin-mediated)[71;72] (Figure 3). All 3 pathways converge at the activation of C3, which continues the activation pathway and/or elicits various biological effects of complement.

Activation of the complement system can have broad specificity, such as occurs with alternative pathway activation, or more specified activation, as occurs with classical and lectin pathway activations. The classical pathway relies on C1 complex activation, which then cleaves C4 and C2, whose cleaved products form a C3 convertase[73]. The C1 complex can be activated in an antibody-dependant manner involving a signal mediated by the globular heads of C1q interacting with immune complexes[74] or in an antibody-independent manner by interactions of proteins, lipids, nucleic acids, and microbial extracts with the collagen-like region of C1q[75]. Examples of classical complement activation by microbes are seen with C1q binding C-reactive protein that binds phosphocholine in the cell wall of *S. pneumoniae*[76] and C1q binding the gp41 fragment of gp160 of HIV-1[77]. Similar to classical pathway activation, lectin pathway activation leads to cleavage of C4 and C2 to form a C3 convertase; however, activation is independent of C1 complexes and instead requires mannan-binding protein (MBP) and MBP-associated serine protease (MASP)[73]. MBP can bind to several carbohydrate structures on a variety of pathogenic micro-organisms and the MBP-MASP complex has been shown to bind bacterial lipopolysaccharides and have bactericidal effects in a complement dependent manner[78]. Additionally, the lectin pathway has been shown to be activated by viruses, such as by MBP binding envelope glycoproteins of HIV-1 and HIV-2[79]. Unlike the classical and lectin pathways, alternative pathway activation occurs by

default. Activation is initiated in the fluid phase by spontaneous and continuous generation of enzymes that cleave C3[80;81], which generates C3b that attaches to host and foreign molecules. Host cells and tissues are protected by an elaborate regulatory system, while foreign molecules, such as any invading pathogen, are marked for phagocytosis[73].

As previously mentioned, all activation pathways converge at the activation of C3. C3 is the most abundant plasma complement protein and activation of C3 is where the activation pathways merge. Protein complexes that make up a protease known as C3 convertase cleave the inactive form of C3 into C3a and C3b. C3a is a potent anaphylatoxin involved in inflammation and chemotaxis and activation of phagocytes[82;83]. C3b can bind to the convertases, thus forming C5 convertases and continue the activation cascade[71]. C3b can also form immune complexes, which can augment antibody responses[84;85], enhance immunologic memory[84], and opsonize cells for removal by phagocytes[86]. Regulation of C3 activation, discussed in more detail below, can occur via the complement regulatory proteins CR1, CD55, and MCP[87].

Considering the key step C3 has in the activation of the complement cascade, it is not surprising that C3 deficiency can have serious deleterious effects. Two case studies in the 1970s by Alper *et al* showed that C3 deficiency or low serum C3 levels due to hypercatabolism resulted in increased susceptibility to repeated infections[88;89]. C3 deficient individuals have also been shown to suffer from autoimmune disorders and recurrent infection, likely resulting from defective immune complex clearance, dimished immune responses, abnormal complement-dependent phagocytosis of opsonized cells, and lack of complement-dependent bactericidal activity as reviewed by Figueroa[90] and Ram[91].

Figure 4: Complement cascade and points of regulation. The complement cascade can be activated by 3 pathways. The classical pathway (initiated by antigen:antibody complexes), the lectin pathway (mediated by mannose-binding lectins), and the alternative pathway (initiated by spontaneous hydrolysis of C3) converge at the point of cleavage of C3 into C3a and C3b. C3b cross-links with C3 convertases to form C5 convertases that cleave C5, which subsequently interacts with other proteins to ultimately form the membrane-attack complex (MAC). The complement regulatory proteins decay-accelerating factor (DAF), membrane cofactor protein (MCP), complement receptor 1 (CR1), and complement receptor 1-related gene/protein Y (Crry) restrict C3 cleavage and deposition, while CD59 inhibits MAC formation.

Figure 4: Complement cascade and points of regulation

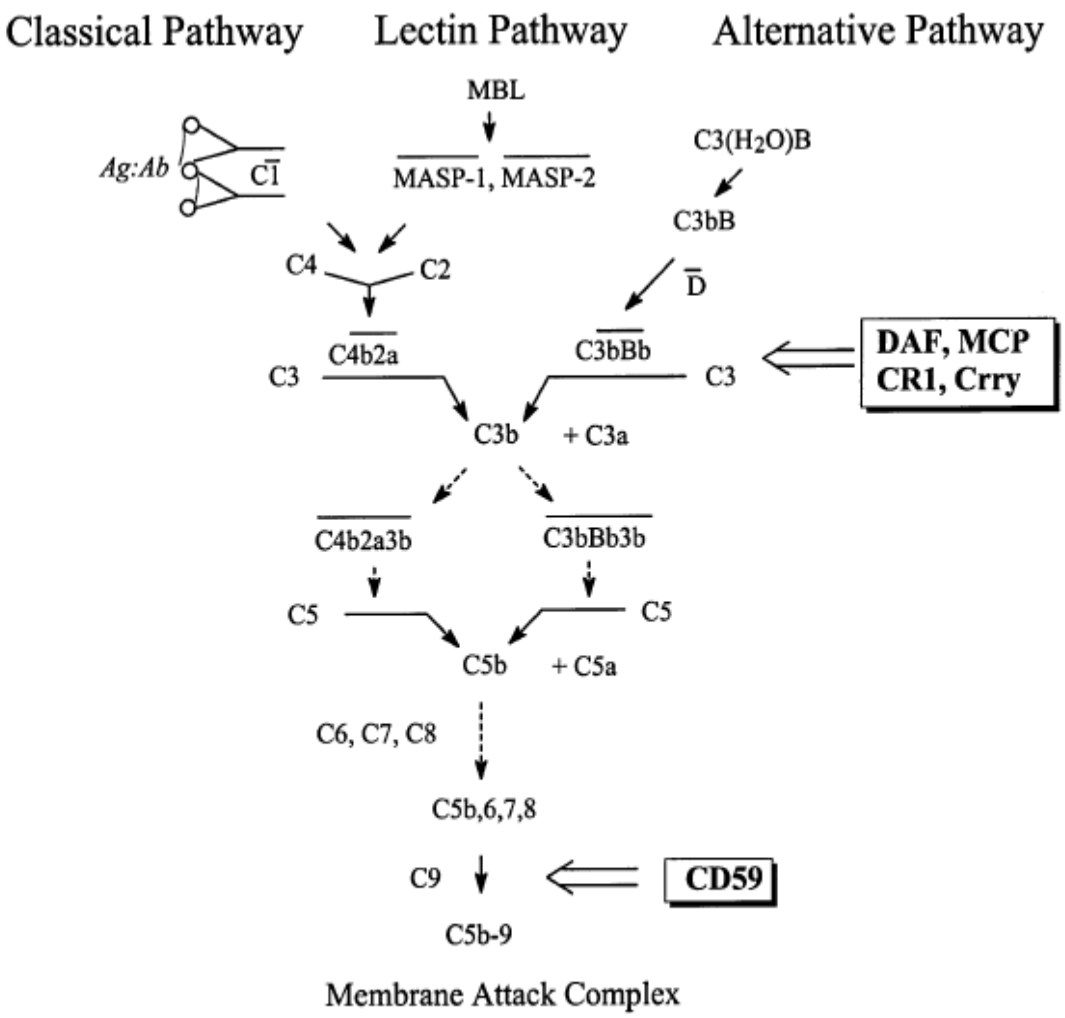


Image taken from Miwa, T. and Song, WC. (2001)[87]

Regulation

While complement is an important component of the immune response, its potent effects require strict regulation. If left unchecked, complement would destroy host cells with the same effectiveness as pathogenic organisms. Regulation is mediated by several membrane-bound and fluid-phase proteins, which function to protect host cells from complement activation and attack[92]. The regulatory proteins function at different points within the complement cascade, primarily at the point of C3 convertase formation/C3 cleavage and at formation of the membrane attack complex (Figure 3). Of the membrane-bound proteins, the major molecules that regulate complement are membrane cofactor protein (MCP/CD46), decay-accelerating factor (DAF/CD55), complement receptor 1 (CR1/CD35), and CD59[91;93].

CD55, MCP, and CR1 regulate complement at the level of C3. CD55 inhibits C3 activation by accelerating the decay of C3 convertases and preventing the formation of new convertases[94]. MCP is a cofactor for factor I-mediated cleavage of C3b[95]; factor I is a soluble regulatory proteinase that cleaves C3b with a cofactor[96]. CR1, the receptor for C3b and C4b in humans, has MCP and CD55 activity. Additionally, CR1 has a role in immune complex processing and clearance[97] . As opposed to the three proteins mentioned above, CD59 regulates complement by preventing assembly of the membrane attack complex[87].

As is the case with complement components, deficiency of regulatory proteins can result in negative effects. Individuals that have deficiencies in CD55 and CD59 suffer from paroxysmal nocturnal hemogloblinuria (PNH)[98;99]. PNH patients experience complement-mediated intravascular hemolysis, which is a result of the regulatory protein

deficiency[100]. Patients with severe malarial anemia have been shown to have an acquired deficiency in CR1 and CD55[34;35;101]. While deficiency of complement regulatory proteins is not a good thing, high levels of regulatory proteins have also been linked to disease. In severe *P. falciparum* malaria, infected red cells form rosettes with uninfected red cells and has been postulated to contribute to CM. Red cells with lower CR1 levels have reduced rosetting[102], providing an example where decreased regulatory proteins could be protective.

Complement receptor-1 related gene/protein Y

In rodents, CR1 does not have the same tissue distribution and function as it does in humans. Search for a CR1 homologue in mice using human CR1 cDNA led to the isoloation of the protein that became known as complement receptor-1 related gene/protein Y (Crry)[103]. Crry is a rodent specific transmembrane protein that is a genetic homologue of CR1[104-106], but not a functional homologue because it does not serve as a receptor for C3b and C4b[107]. However, Crry regulates complement and has CD55 and MCP activiity[104;108;109]. Crry has been found to be critical during embryonic development[110] and is key in maintaining homeostasis of the complement system[111]. While the function of Crry in mice overlaps with that of mouse CD55, Crry has been found to be more important in the protection of erythrocytes from complement-mediated attack[110;112].

Mouse CR1 is not found on the surface of erythrocytes[113], unlike human CR1. However, both Crry and human CR1 have MCP and CD55 activity; therefore, it is possible the CR1 on human red cells is functioning identically to Crry on mouse red cells.

If this is the case, correlations between findings with Crry in mice and CR1 in humans can be made in order to understand pathogenic mechanisms occurring in human malaria infection.

Animal Models for Studying Malaria

There are many facets of a malaria infection that can be studied and several tools that exist for such studies. Human malaria parasites can be studied *in vitro* by culturing and aspects of infection can be examined by various epidemiological studies. However, animal models provide a useful platform for elucidating pathogenic mechanisms involved in the development of severe malarial disease. There are models for studying all stages of the parasite life cycle; however, for the context of this thesis I will focus on models involving only the blood stage parasites.

Mouse Models

There are several mouse models of malaria infection, which differ in virulence and disease presentation depending upon the strain of animal and species of *Plasmodium* parasite used[114-116] (Table 1). The study of CM and SMA are of particular interest with these mouse models since those severe manifestations have a high mortality rate.

Table 1: Malaria infections is different mouse strains

Plasmodium	Strain/clone	Mouse strain	Lethal infection	Experimental use	CM	Anemia
chabaudi chabaudi	AS	C57Bl/6 BALB/c CBA	No	Immune mechanisms, pathogenesis	No	Yes
		A/J DBA/2	Yes		No	Yes
chabaudi adami	556KA	C57Bl/6 BALB/c C3H	No	Immune mechanisms, pathogenesis	No	Yes
berghei	ANKA	C57Bl/6 BALB/c CBA	Yes	Pathogenesis of CM	Yes	Yes
		DBA/2J	Yes	Resolving CM	Yes	Yes
	K173	BALB/c DBA/2J CBA/T6	Yes	Non CM control	No	Yes
		C57Bl/6	Yes	Pathogenesis of CM	Yes	No
yoelii	17X	BALB/c Swiss	Yes	Pathogenesis of CM	Yes	Yes
		C57Bl/6 BALB/c CBA	No	Immune mechanisms, pathogenesis	No	Yes
	YM	C57Bl/6 BALB/c CBA DBA Swiss	Yes	Vaccines	No	
vinckei vinckei		BALB/c	Yes	Chemotherapy, immune mechanisms, pathogenesis	No	Yes

Modified from Li et al[116] and Langhorne et al[115]

Specific components of the immune response that may be involved in the pathogenesis of these severe manifestations have been studied with the use of inbred strains of mice.

Since the rodent models vary drastically based on infecting species, this section focuses on certain aspects per species within a particular animal breed; for a more complete listing see table 1. The rodent parasites most commonly used to study anemia are *P. chabaudi, P. berghei, P. vinckei,* and *P. yoelii*[28]. The virulence of each of these species varies depending on the strain of mouse used *P. berghei* and *P. vinckei* are lethal in all strains of mice, while *P. chabaudi* and *P. yoelii* are only lethal in some strains[115;117]. *P. berghei* ANKA causes death from cerebral complications in C57BL/6 and CBA mice before severe anemia develops and is lethal in CD-1 mice with the development of anemia[114;118]. We have observed C57BL/6 mice infected with *P. berghei* ANKA develop parasitemia within a week following infection with 10^6 parasitized red blood cells; the animals develop cerebral complications, such as hind leg paralysis and seizures, and die without the evidence of severe anemia. On the other hand, *P. chabaudi* AS infection causes severe anemia with hyperparasitemia of 30 to 40%, differing in lethality depending upon the strain of mouse used[119;120]. C57BL/6 mice infected with 10^6 *P. chabaudi* AS-parasitized red blood cells develop a peak parasitemia approximately 8 days post-infection with a severe decline in RBC count, that then recovers with a corresponding decline in parasitemia and mice survive. However, in A/J mice given the same infectious dose of *P. chabaudi* AS, mice develop a peak parasitemia and severe decline in RBC count at the same time as the C57BL/6 animals, but proceed to die from the severe anemia caused by the infection[119]. The commonality among the various mouse

models is they all differ significantly from the clinical picture of severe anemia seen with *P. falciparum*.

Evans *et al*.[121] described a model of SMA by *P. berghei* ANKA infections in semi-immune BALB/c mice that has similarity to *P. falciparum* infection. The animals developed severe anemia in the presence of a low parasite burden. Semi-immune BALB/c mice, generated through repeated rounds of *P. berghei* infection and drug cure, were infected with 10^4 *P. berghei* ANKA parasitized red blood cells via intraperitoneal injection. The model consisted of animals that developed severe anemia, evidenced by a rapid decline in hemoglobin levels, while having a patent infection that peaked at approximately 1%. The reticulocyte levels in these animals elevated to approximately 35% 2 days after the anemic crisis and then returned to basal levels when the animals recovered from the anemia. They also demonstrated an accelerated destruction of uninfected red cells, which has been reported in humans infected with *P. falciparum*[22].

Non-human Primate Models

In addition to rodent models, there are non-human primate models of malarial infection. Semi-immune *Aotus* monkeys infected with *P. falciparum* have been used to study severe malarial anemia. *Aotus* monkeys previously vaccinated and exposed to an initial malaria parasite are infected with *P. falciparum* FVO strain[122]. The monkeys experience various outcomes during the second infection which include: no detectable parasites and anemia; no detectable parasites microscopically, but PCR positive and severe anemia; low level parasitemia and moderate anemia; low level parasitemia and severe anemia; and parasitemia equal to or greater than 5% and severe anemia. The

major advantage of this model is the use of *P. falciparum* in an animal that is similar to humans. However, since monkeys are not inbred laboratory animals, genetic modifications to study varying host factors cannot be made. The largest deterrent in using non-human primate models is the cost and very limited availability.

Hypotheses and Specific Aims

To better understand severe malarial anemia, our lab investigates the role of complement and complement regulatory proteins in the pathogenesis of SMA. **Our central hypothesis is that deficiencies in complement regulatory proteins predispose uninfected erythrocytes to complement-mediated damage and destruction during a malaria infection.** To test the central hypothesis, my project was divided into the following specific aims:

Specific Aim 1 – To determine whether the mouse complement regulatory protein Crry protects erythrocytes from complement-mediated attack during a malaria infection.

I specifically hypothesized that deficiencies in Crry would result in more red cell destruction evidenced by greater erythrophagocytosis.

Specific Aim 2 – To determine whether the complement component C3 is essential for the destruction of red cells during a mouse malaria infection.

I hypothesized that deficiency in C3 would result in less severe anemia during a malaria infection.

Specific Aim 3 – To develop a relevant C57BL/6 mouse model of severe malarial anemia

A primary criticism of current rodent models is the development of hyper-parasitemia, which is not seen in human infections. Additionally, the high parasitemia levels seen in available C57BL/6 mouse models may also mask what is happening to uninfected red cells in an infected host. Therefore, my goal was to develop a mouse model where severe malarial anemia develops in the presence of a low level parasitemia. I hypothesized that infection after a prior immune response to Plasmodia would result in increased severity of anemia during a malaria infection.

Chapter 2

Materials and Methods

Introduction

The lab is interested in understanding pathogenic mechanisms involved in the development of severe malarial anemia. Since symptomatic disease in malaria is seen during the blood stage of the *Plasmodium* life cycle, we designed all experimental procedures to utilize the blood stage of the parasite only. It was also important to understand the infection in the context of a host-pathogen setting; therefore, we utilized two different rodent models of malaria infection that resulted in severe anemia. During the course of infection we monitored multiple parameters described below, and we utilized various techniques to characterize and elucidate potential mechanism(s) involved in the development of malaria in this model system.

Mice

Mice were used under protocols approved by the Institutional Animal Care and Use Committees (IACUC) of the Uniformed Services University of the Health Sciences and of the Pennsylvania State University College of Medicine. C57BL/6 mice were purchased from Jackson Laboratories. Subsequently, $Crry^{-/-}$ and $C3^{-/-}$ mice, both in a C57BL/6 background, were bred in house after receiving initial breeding pairs from collaborators. $Crry^{+/-}$ mice were generated by crossing $Crry^{-/-}$ mice with C57BL/6 wild-type mice. All mice used in the experiments, both male and female, were 6-12 weeks of

age at the time of the initial infection. Mice were kept in a pathogen free barrier facility until initiation of the experiments.

Malaria Parasites and Infection

Two different species of *Plasmodium* were used throughout this work, *Plasmodium chabaudi chabaudi* and *Plasmodium berghei*. *Plasmodium chabaudi chabaudi* AS parasites were obtained from David Walliker at the University of Edinburgh. *Plasmodium berghei* ANKA parasites were a gift from Martha Sedegah at the Walter Reed Army Institute of Research. *Plasmodium chabaudi chabaudi* AS and *Plasmodium berghei* ANKA parasites were maintained as stocks in glycerolyte (Baxter, Deerfield, IL) in liquid nitrogen. Prior to an experiment, stocks were expanded by intraperitoneal (IP) injection into mice. When the parasitemia was 10-15%, as determined by Giemsa-stained thin blood smear, cardiac blood anticoagulated with citrate phosphate dextrose (Sigma-Aldrich, St. Louis, MO) was obtained and diluted to a concentration of 5×10^6 infected red blood cells (IRBCs)/mL in RPMI-1640 medium containing 2% bovine serum albumin (BSA; Sigma-Aldrich). Unless otherwise stated, 10^6 IRBCs were injected IP into each mouse to start an experimental infection. At designated time-points, 20-40 µL of tail vein blood was collected into EDTA capillary tubes (Heska Corp., Loveland, CO) and Giemsa-stained thin blood smears were prepared directly from a droplet of tail blood. Reticulocyte smears were prepared after mixing 3 µL of whole blood with 2 µL of reticulocyte stain (Sigma-Aldrich). For collection of serum or plasma, blood was obtained from the retro-orbital plexus into an EDTA-coated or uncoated Pasteur pipet. Following euthanasia, livers and spleens were obtained,

weighed and fixed in 10% buffered formalin for histochemical processing. Red blood cell concentrations were determined using a hemacytometer or a Mindray BC-2800 vet hematology analyzer (Mindray Bio-Medical Electronics Co., P.R. China). A minimum of 500 total red blood cells were counted to determine percent parasitemia and reticulocytes.

Crry and C3 Studies

For the complement studies, C57BL/6, $Crry^{+/-}$, and $C3^{-/-}$ animals were inoculated with either *P. chabaudi* AS or RPMI-1640 medium as a sham control. Mice were analyzed as described above, and RBCs from these animals were used in transfer assays described below.

Anemia Model Studies

C57BL/6 animals were inoculated with *P. chabaudi* AS. At 5 days post-infection with *P. chabaudi* AS a Giemsa-stained thin blood smear was prepared directly from tail blood and the parasitemia was determined to confirm that all animals were infected. Mice were then allowed to continue through the entire course of infection without any further handling. At approximately 6-8 weeks post *P. chabaudi* infection, tail blood was again obtained to ensure that the parasitemia was cleared and, if so, mice were then inoculated with either *P. berghei* ANKA or RPMI-1640 medium as a sham control. Additionally, a group of naïve C57BL/6 mice were inoculated with *P. berghei* ANKA for comparison. Mice were analyzed as described above.

Flow Cytometry

Measurement of RBC surface Crry

Flow cytometry was used to assess the amount of Crry on the surface of RBCs. All staining was performed in a 96-well plate. Purified rat anti-mouse Crry/p65 (BD Pharmingen, San Jose, CA) and rat IgG_1 (BD Pharmingen), each diluted 1:50 in wash buffer (phosphate buffered saline (PBS; Sigma-Aldrich) containing 1% BSA), were used as primary antibodies. DyLight 488 labeled goat anti-rat IgG (KPL, Gaithersburg, MD) diluted 1:400 in wash buffer was used as a secondary antibody. In each well 3 μL of whole blood was mixed with 100 μL Alsever's solution (Sigma-Aldrich) and 100 μL wash buffer. Cells were spun down at 1500 rpm for 5 minutes and the supernatant was discarded. RBC pellets were resuspended in 100 μL of 1 μg/mL Hoechst (Sigma-Aldrich) and incubated for 30 minutes at 37°C protected from light. Cells were pelleted as above and then resuspended in 100 μL of appropriately diluted primary antibody and incubated at room temperature for 30 minutes protected from light. Each well then received 100 μL of wash buffer and cells were spun at 1500 rpm for 5 minutes and the supernatant was discarded. Cells were then washed with 200 μL wash buffer and pellets were resuspended in 100 μL of appropriately diluted secondary antibody and incubated for 30 minutes at room temperature, again protected from light. Cells were washed and resuspended in PBS containing 2% paraformaldehyde (Sigma-Aldrich) and stored at 4°C.

Samples were acquired on a BD LSRII flow cytometer using BD FACSDiva software (BD Biosciences, San Jose, CA). Analysis was performed using FCS Express (version 3) software (De Novo Software, Thornhill, Ontario, Canada). For the analysis, RBCs were gated based upon their forward and side scatter properties. RBCs were then

further gated into infected and uninfected populations using the Hoechst histograms. Additionally, infected and uninfected RBC populations were evaluated for Crry expression.

Ki-67 and Ter-119 characterization of mouse spleen

Spleens harvested from animals at the time of euthanasia were placed into 5 mL cold PBS and crushed with the flat end of a 3 mL syringe plunger. The resulting cell mixture was strained through a 40 μm cell strainer and centrifuged at 400 g to pellet cells. Pellets were then washed with 5 mL cold PBS and resuspended in 1 mL RBC lysis buffer (Sigma-Aldrich) and incubated at room temperature for 10 minutes until RBCs were fully lysed. Cells were mixed with 5 mL PBS and pelleted as described above. Cell pellets were washed with 5 mL PBS and resuspended in 1 mL PBS containing 1% BSA. The cells were then counted using a hemacytometer and preparations of 10^7 cells/mL were made for each spleen.

For detection of Ki-67 (a marker for proliferation) and Ter-119 (an erythroid lineage marker), approximately 10^6 cells were fixed in 100 μL of 4% paraformaldehyde in PBS for 30 min at room temperature. Following fixation, the cells were washed once with PBS containing 2% BSA (FACS buffer) and stored in the same buffer overnight at 4 °C. The following day, the cells were permeabilized by incubation in permeabilization buffer (PBS containing 2% BSA and 0.5% saponin (Sigma-Aldrich)) at room temperature for 10 minutes. This was followed by incubation for 20 minutes at room temperature in a 1:50 dilution of FITC-labeled mouse anti-Ki67 (BD Biosciences) in permeabilization buffer, washed once in FACS buffer, and incubated for 30 minutes in a 1:50 dilution of

PE-labeled rat anti-mouse TER-119 (BD Biosciences). Finally, the cells were resuspended in cold 1% paraformaldehyde in PBS. Analysis was performed on an LSRII flow cytometer using FACS Diva software.

Western Blotting

Verification of malaria antigen

Malaria antigen was prepared as described in the quantitation of parasite-specific antibody section found below. For Western analysis, 500 ng total protein was loaded per lane on a 4-12% Bis-Tris acrylamide gel (Invitrogen, Carlsbad, CA) and transferred to a nitrocellulose membrane. Membranes were blocked with 1% IgG-free BSA (Jackson ImmunoResearch, West Grove, PA) in tris-buffered saline (TBS, pH=8.0) containing 0.05% Tween-20. Plasma from an immune mouse (*P. chabaudi/P. berghei* 20 days post-infection) was used as a source of primary antibody. Horseradish peroxidase conjugated goat anti-mouse IgG (KPL, Gaithersburg, MD) was used as the secondary antibody. Membranes were developed with the Pierce ECL western blotting substrate (Thermo Scientific) and visualized on the Fuji LAS-3000 with ImageReader software.

Evaluation of Cytokines

Inflammatory/anti-inflammatory

In order to assess the inflammatory response, a mouse cytokine 10-plex panel was used according to the manufacturer's instructions (Invitrogen, Carlsbad, CA) to determine concentrations of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 p40/70, GM-CSF,

IFN-γ, and TNF-α in plasma samples. Samples were run on a Luminex 100 System using Masterplex QT software.

EPO

An MSD® MULTI-ARRAY® mouse EPO assay was used according to the manufacturer's instructions (Meso Scale Discovery, Gaithersburg, MD) to determine concentrations of erythropoietin, a cytokine that regulates red cell production, in plasma and serum samples. Samples were analyzed on an MSD Sector Imager 2400A.

Quantitation of Parasite-specific Antibodies

Antigen preparation

For malaria antigen extraction, the procedure was a modification of that previously reported[123]. Briefly, approximately 500 µL of citrated cardiac blood was obtained from two mice infected with *P. chabaudi* AS or *P. berghei* at ~20% parasitemia, consisting of mostly the ring stage of the parasite. Each sample was divided into two equal samples, one for immediate lysis and the other for overnight culture in order to obtain late trophoizes and schizonts. The samples for lysis were centrifuged at 2,500 g for 5 minutes. The pellet was then resuspended in PBS with 0.1% saponin and 1x protease inhibitor cocktail (Sigma-Aldrich). The suspensions were incubated on ice for 5 minutes. The samples were centrifuged again and the pellets were then washed two more times in the same lysis solution and resuspended in 500 µL of PBS containing 1x protease inhibitor. A Giemsa-stained smear confirmed the presence of intact parasites and ghost red cells. The samples were then frozen at -20 °C overnight, and the following day they

were thawed and sonicated for 5 minutes. This was followed by another cycle of freezing, thawing, and high speed vortexing. Finally, the samples were cleared by pulsed centrifugation at 14,000 rpm and the supernatants were removed and stored at -20 °C. The samples for overnight culture were added to 5 ml of RPMI 1640 (Sigma-Aldrich) containing 25 μg/ml gentamicin and were incubated overnight in a low O_2 atmosphere at 37 °C. The following day, the samples were processed exactly as described above. The total protein concentrations were measured using the bicinchonic acid method (Thermo Scientific,Rockford, IL).

ELISA

Parasite-specific IgG in mouse serum was measured with an ELISA. Flat-bottom 96-well Immulon 2 HB ELISA plates (Dynex Technology Inc., Chantilly, VA) were coated with 100 ng per well of either *P. chabaudi* or *P. berghei* antigen diluted with PBS overnight at 4°C. Plates were then blocked with 200 μL/well of blocking buffer (5% Blotto non-fat dry milk (Santa Cruz Biotechnology Inc., Santa Cruz, CA) in TBS containing 0.1% Tween-20 (Sigma-Aldrich)) for 2 hours at room temperature. Sera that had been diluted 1:1000 in dilution buffer (0.1% BSA in TBS containing 0.1% Tween-20) were added to each well and incubated at room temperature for 1 hour. After extensive washing in wash buffer (TBS containing 0.1% Tween-20), 0.1 μg/well of peroxidase-labeled goat anti-mouse IgG (H+L) (KPL, Gaithersburg, MD) was added and incubated at room temperature for 1 hour. Plates were washed extensively and then 100 μL of ABTS peroxidase substrate (KPL) was added to each well and incubated at room temperature for 30 minutes. The reaction was stopped by addition of 100 μL/well of a

1% sodium dodecyl sulfate (Sigma-Aldrich) solution. Absorbance at 405 nm was read with an Emax precision microplate reader (Molecular Devices, Sunnyvale, CA) using SoftMax Pro (version 4.3) software. All sera samples were run in duplicate.

Quantitation of Erythrophagocytosis

H&E counts

Quantitation of erythrophagocytosis in the liver was determined by examination of hematoxylin and eosin (H&E) stained tissue sections and by immunohistochemical staining for macrophages in liver sections. Formalin-fixed paraffin embedded liver sections were stained with H&E and then examined under 40x magnification. Macrophages were identified as cells present in the liver sinusoids containing cytoplasmic hemozoin and an exocentric nucleus. The number of macrophages with internalized RBCs were counted in 20 consecutive fields, and the average number of macrophages with internal RBCs per high power field was calculated. The microscopist was always blinded to the experimental group assignment of each animal.

F4/80 Immunohistochemistry

In order to assess the percent phagocytosis in the liver and verify our finding from the H&E stains, we examined the macrophage marker F4/80 through immunohistochemistry. For immunohistochemical staining, formalin-fixed paraffin embedded liver sections were deparaffinized and rehydrated by standard methods. Antigen retrieval was performed with 1 mM EDTA (Sigma-Aldrich) at 100°C in a steamer for 20 minutes. Endogenous peroxidase activity was blocked using a 3%

peroxide solution (Sigma-Aldrich) and endogenous biotin activity was blocked using an Avidin/Biotin blocking kit (Vector, Burlingame, CA). Rat anti-mouse F4/80 diluted 1:25 (a kind gift from Dr. Christopher Norbury) or rat IgG isotype control diluted 1:25 (BD Biosciences, San Jose, CA) was used as primary antibodies, followed by a biotin-conjugated goat anti-rat IgG diluted 1:50 (BD Biosciences) that was used as the secondary antibody. HRP-conjugated streptavidin (BD Biosciences) was added after the secondary antibody. All antibody dilutions were in antibody diluent (Dako, Carpinteria, CA) and all staining incubations proceeded for 30 minutes at room temperature in a humidity chamber. Sections were developed with DAB chromogen (Vector). Counterstaining was performed with Giemsa (Sigma-Aldrich) for 45 minutes, followed by a 2 minute destain in an acetic acid wash. A minimum of 100 macrophages, identified by positive F4/80 staining, were counted to determine percent erythrophagocytosis.

In vitro *Erythrophagocytosis*

We also wanted to assess erythrophagocytosis *in vitro*. To this end, J774A.1 macrophages were maintained in macrophage media [Dulbecco's modified eagle medium (DMEM; Invitrogen) containing 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 1% Glutamax (Invitrogen), 1% non-essential amino acids (Sigma-Aldrich), 1% sodium pyruvate (Invitrogen), and 1% penicillin/streptomycin (Atlanta Biologicals)] in 25 mL cultures at 37°C with 5% CO_2. The day prior to the assay, macrophages were seeded at density of 10^5 cells/well in 24-well cell culture plates and then stimulated with 1 μg/mL LPS in macrophage media overnight. Two hours prior to adding RBCs to macrophages, negative control wells were incubated with 50 μM

cytochalasin D (Invitrogen) in macrophage media, in order to inhibit actin polymerization. Glycerolyte preserved RBCs were thawed with sodium chloride as previously reported[123] and pooled within each experimental group. For each group a 5% hematocrit RBC solution in PBS was made and then labeled with CFSE (Invitrogen) at 10 μM in PBS for 15 minutes at room temperature with constant mixing. Labeled RBCs were subsequently washed 3 times with PBS and then a subset of labeled RBCs was coated with a Ter-119 antibody to create a positive control. The positive control cells were made by mixing 100 μL labeled RBCs with 400 μL 10 mM EDTA (Sigma-Aldrich) in PBS and 1 μL rat anti-mouse Ter-119 (eBioscience), which was then incubated at room temperature for 30 minutes with constant mixing. Cells were washed 3 times with PBS and resuspended in 1 mL PBS. All labeled RBC preparations were counted with a hemacytometer and adjusted to a concentration of 2×10^6 cells/mL in macrophage media. Media was removed from the prepared macrophage cultures and 10^6 of the appropriate RBC in macrophage media were added to each well and incubated at 37°C with 5% CO_2 for 4 hours with constant mixing. Each experimental condition was performed in triplicate. Macrophages were washed 3 times with macrophage media and then external RBCs were lysed with RBC lysis buffer (Sigma-Aldrich). Cells were washed, detached with cold macrophage detachment media (PBS containing 100 mM EDTA), and fixed with cold 2% paraformaldehyde in PBS. Cells were stored at 4°C in the dark until acquisition, which was performed on an LSRII flow cytometer using FACS Diva software. The percent of $CFSE^+$ cells was determined compared to a macrophage only control.

Single-chain Antibody Red Cell Augmentation of Surface Crry

Single-chain Fab antibody connected to Crry protein (scFV-Crry) was used to increase the surface level of red cell Crry. Single-chain TER-119 Fab was prepared using a Fab preparation kit (Thermo Scientific) with rat anti-mouse Ter-119. Three days prior to infection a subset of mice were injected IP with 500 µL/mouse scFV-Crry or anti-Ter-119 Fab as a control. Mice received a second dose identical to the first injection at 3 days post infection. Blood was obtained from the tail vein the day following injection and was stained for Crry as outlined above to verify an increase in surface Crry levels.

Ex vivo Labeling of RBC's

In order to perform RBC survival studies the RBCs first needed to be labeled *ex vivo*. IRBCs were obtained from C57BL/6 *P. chabaudi*-experienced mice that were recently infected with *P. berghei*; uninfected RBCs were obtained from C57BL/6, $Crry^{+/-}$, and $Crry^{-/-}$ naïve animals. CPD anticoagulated cardiac blood was collected and RBCs were labeled *ex vivo* with either DiD or DiI following the manufacturer's recommendations (Invitrogen).

RBC Survival Studies

Transfer of $Crry^{+/-}$ and $Crry^{-/-}$ RBCs

Following labeling, an equal volume of DiI-labeled wild-type RBCs and DiD-labeled wild-type, $Crry^{+/-}$, or $Crry^{-/-}$ RBCs were mixed together. Mice were sedated with Ketamine/Xylazine and 200 µL of mixed labeled RBCs was injected into a retro-orbital plexus. At pre-defined intervals, EDTA-anticoagulated tail blood was collected and 5 µL

was added to 200 μL of 2% paraformaldehyde in PBS. The samples were stored at 4 °C in the dark until acquisition, which was performed on an LSRII flow cytometer using FACS Diva software. RBCs were gated on the basis of the forward and side scatter characteristics using logarithmic amplification. The percent positive cells in FL2 and FL3 were determined in comparison to an unstained control.

RBC transfer into anemia model animals

After labeling, the RBCs were resuspended at a concentration of 5 x 10^9 RBCs/ml in RPMI 1640. After sedation with Ketamine/Xylazine, mice were injected with 100 μL of labeled RBCs into a retro-orbital plexus. At pre-defined intervals, EDTA-anticoagulated tail blood was collected and 5 μL was added to 200 μL of 2% paraformaldehyde in PBS. The samples were stored at 4 °C in the dark until acquisition, which was performed as above using a 635 nm in line laser. RBCs were gated on the basis of the forward and side scatter characteristics using logarithmic amplification. The percent positive cells in FL3 was determined in comparison to an unstained control.

In vivo *Imaging*

Animals that received labeled RBC transfers also had their abdomens imaged to see if there were specific organs involved in the RBC clearance from the peripheral blood. Five days post RBC transfer, animals were euthanized with Ketamine/Xylazine and their abdominal cavities were surgically opened to expose the organs. Animals were placed in a Xenogen IVIS 50 Imaging System (Caliper Life Sciences, Hanover, MD) and

a fluorescent image was taken. Living Image software (version 3.1) was used to capture and process all images.

Statistical Analysis

All data, unless otherwise noted, are presented as mean ± standard deviation (SD). Statistical analysis was performed using the GraphPad Prism 4 software (GraphPad Software, Inc., La Jolla, CA). Comparison of means between two groups was performed using a t-Test with the Welch's correction. All tests were two-tailed with a significance level of 0.05.

Chapter 3

Partial deficiency of the red cell complement regulator Crry leads to increased erythrophagocytosis during malaria infection of mice

Introduction

Plasmodium falciparum is an intracellular parasite of humans responsible for 1-2 million deaths per year[3]. The majority of these deaths occur in children from Sub-Saharan Africa and are due to complications such as cerebral malaria and severe malaria-associated anemia (SMA). The pathogenesis of severe anemia is not well understood, but is likely due to multiple factors. One contributory factor is the accelerated destruction of uninfected red cells independent of the level of parasitemia[59;124;125]. Jakeman *et al.*[22] used mathematical modeling on data from patients infected with *P. falciparum* to treat neurosyphilis and showed that an average of 8.5 uninfected red cells are destroyed for every parasitized red cell. Additionally, patients treated for *P. falciparum* malaria continue to experience red cell destruction after treatment, indicating the parasite is not directly responsible for the destruction of red cells resulting in anemia[126]. However, the mechanism(s) by which uninfected red cells are destroyed at an accelerated rate during malaria infection is/are unknown. We suspect that complement activation during malaria infection plays an important role in this process.

Complement is a component of the innate immune system, comprised of more than 30 plasma and membrane bound proteins. The complement system can elicit various biological effects including opsonization of cells, which can then be cleared from the circulation by phagocytic cells.[71] Complement regulatory proteins provide protection

to host cells from autologous complement activation such as can take place during malaria infections. We have shown that children with severe malaria-associated anemia have lower levels of the red cell complement regulatory proteins complement receptor 1 (CR1/CD35) and decay-accelerating factor (DAF/CD55)[35].

To understand the significance of complement regulatory protein deficiencies in the pathogenesis of SMA we studied the role of complement receptor 1-related gene/protein Y (Crry) in red cell protection during *Plasmodium chabaudi* infection in mice. Crry is a rodent-specific transmembrane protein that is involved in the regulation of C3 activation[87;104]. It is a genetic homologue of human CR1; however, Crry is considered a functional homologue of human DAF and membrane cofactor protein (MCP/CD46) due to its decay-accelerating activity and ability to serve as a cofactor for factor I-mediated cleavage of C3b and C4b[108]. Crry is widely distributed in the mouse, including being present on the surface of erythrocytes. Additionally, Crry has been shown to be responsible for maintaining homeostasis of the complement system[127]. In the study presented here, we utilized *P. chabaudi*-infected C57BL/6 wild-type and Crry heterozygous mice to determine whether the complement regulator Crry was important in protecting red cells from erythrophagocytosis during a malaria infection.

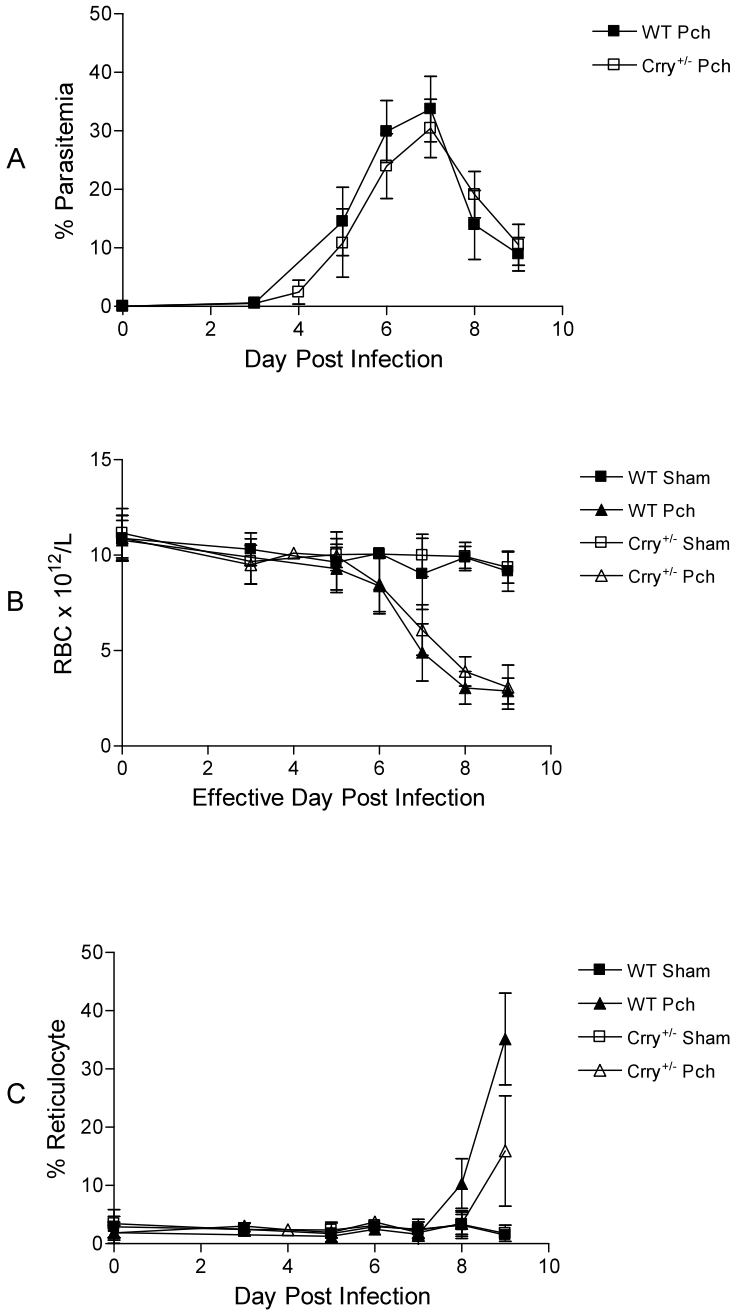
Results

Course of malaria infection in Crry$^{+/-}$ mice

In order to explore the role of Crry in protection of red cells during infection we infected Crry heterozygote (Crry$^{+/-}$) and C57BL/6 mice with *P. chabaudi*. There was no difference in the course of parasitemia during infection nor was there a difference in the

Figure 5: Comparison of parasite, RBC, and reticulocyte kinetics during infection in wild-type and Crry$^{+/-}$ mice. Wild-type (WT) and Crry$^{+/-}$ mice were infected with *P. chabaudi* (Pch) or RPMI/BSA as a sham control. Average parasitemia (A), RBC counts (B), and reticulocyte levels (C) were monitored throughout the course of infection. Data are pooled from 4 independent experiments and normalized such that day 0 is the baseline values and day 7 is the peak of parasitemia.

Figure 5: Comparison of parasite, RBC, and reticulocyte kinetics during infection in wild-type and Crry$^{+/-}$ mice



RBC counts between infected wild-type and Crry$^{+/-}$ mice (Figure 5A and B). All infected animals had an increase in reticulocytes; however, wild-type mice had a stronger reticulocyte response following the nadir of the anemia (Figure 5C). Necroscopic evaluation revealed that the liver and spleen in infected wild-type and Crry$^{+/-}$ mice were enlarged (Figure 6)

Changes in surface Crry during malaria infection

To further evaluate the role of Crry in red cell protection we measured the expression of Crry on the surface of uninfected RBCs from infected and uninfected wild-type and Crry$^{+/-}$ mice. FACS analysis of the uninfected RBCs showed infected mice had a decline in surface Crry levels compared to baseline and compared to their sham counterparts (Figure 7). Conversely, sham infected animals had an increase in surface Crry expression compared to baseline values (Figure 7), which is likely due to an increase in reticulocyte levels resulting from multiple bleeds. As expected, the level of RBC surface Crry in Crry$^{+/-}$ mice was half that of wild-type.

Figure 6: Comparison of organ weights between Crry$^{+/-}$ and wild-type animals. The spleen and liver were obtained from wild-type and Crry$^{+/-}$ mice, either sham infected (white bars) or infected with *P. chabaudi* (black bars), at day 8-9 post infection. Average weight normalized to the percent of the total body weight ± SD is shown for each group. *P. chabaudi*-infected animals have enlarged livers and spleens.

Figure 6: Comparison of organ weights between wild-type and Crry$^{+/-}$ animals

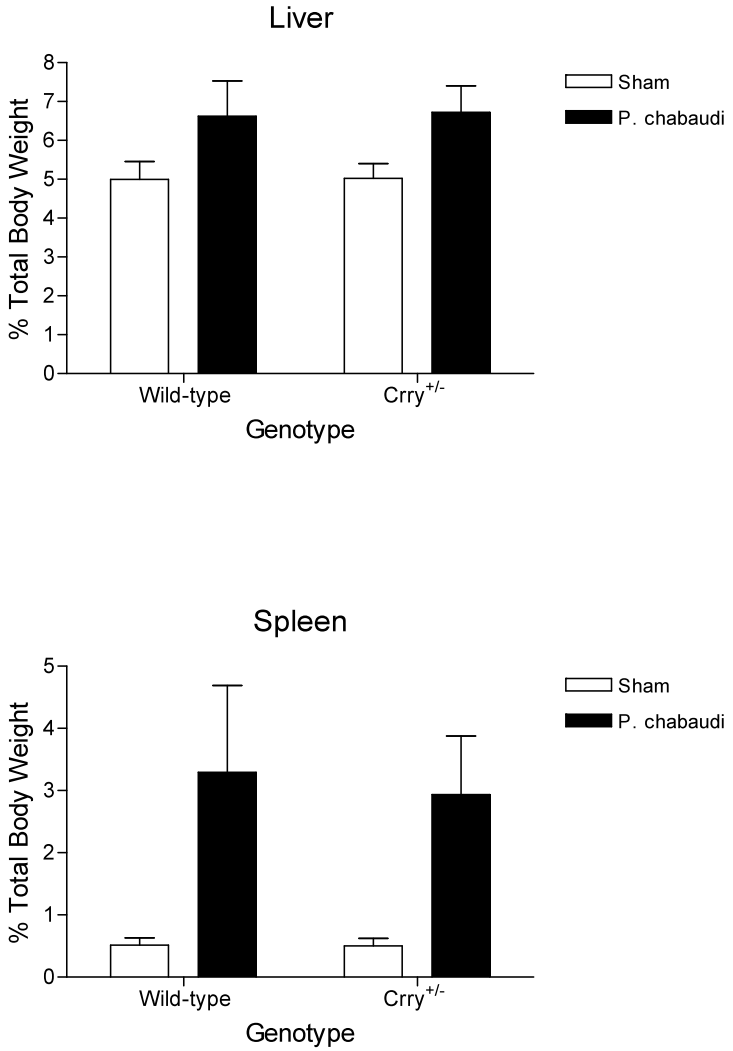
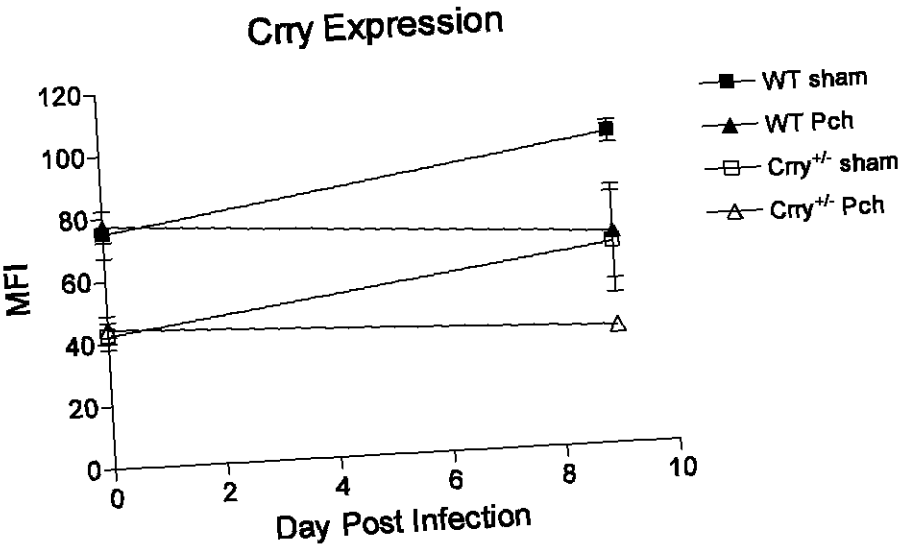


Figure 7: Crry expression during a *P. chabaudi* infection. Wild-type (WT) and Crry$^{+/-}$ mice infected with *P. chabaudi* (Pch) or RPMI/BSA as a sham control had the surface levels of Crry on RBCs measured by flow cytometry. The uninfected RBC population within each animal was gated based upon negative Hoechst staining and analyzed for Crry expression. Expression levels in malaria infected mice declined, while sham infected animals increased over baseline values.

Figure 7: Crry expression during a *P. chabaudi* infection

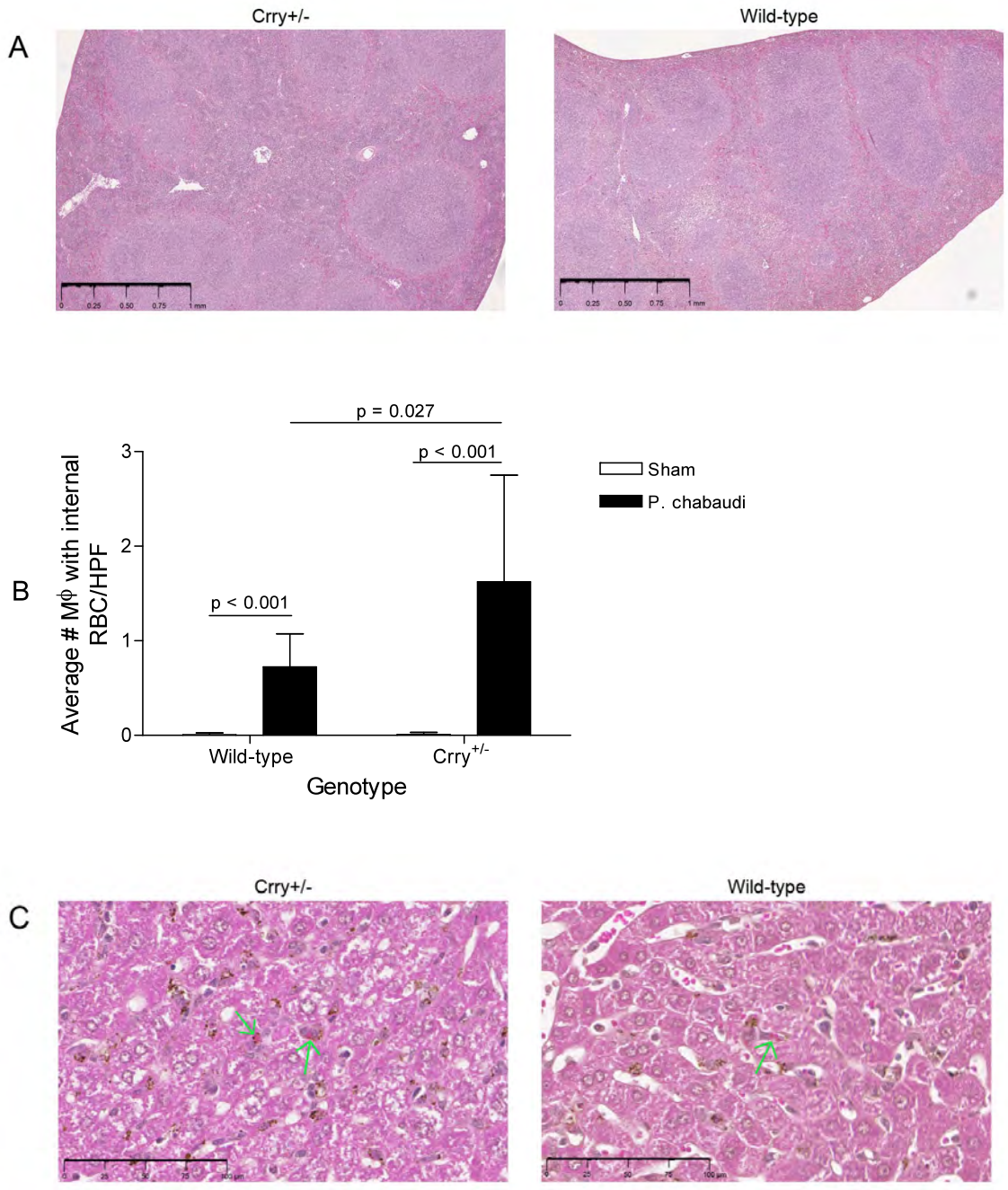


Crry$^{+/-}$ mice have increased red cell destruction and evidence of extramedullary hematopoiesis

To gain a better understanding of the organ enlargement and investigate potential mechanisms involved in the development of anemia, we examined the histopathology of the spleen and liver of wild-type and Crry$^{+/-}$ mice. H&E tissue sections of livers from infected Crry$^{+/-}$ and wild-type animals revealed erythrophagoyctosis (Figure 8C). Macrophages in liver sections from infected Crry$^{+/-}$ mice often had multiple internalized red cells (Figure 8C), which was not seen with the infected wild-type mice. Quantitation of the observed erythrophagocytosis revealed a significant increase in erythrophagocytosis in infected Crry$^{+/-}$ animals as compared to wild-type animals (Figure 8B). H&E stained spleen sections revealed a massive expansion of the red pulp by early erythroid lineage cells, which was greater in Crry$^{+/-}$ animals than in wild-type animals (Figure 8A).

Figure 8: Histopathology of the liver and spleen of wild-type and Crry$^{+/-}$ *P. chabaudi*-infected mice. (A) Representative H&E stained spleen section at 5X magnification from a wild-type (right) and Crry$^{+/-}$ (left) *P. chabaudi*-infected animal. (B) Quantitation of erythrophagocytosis in the liver shown as the average number of macrophages with internal RBCs per high power field ± SD. *P. chabaudi* infected animals had significantly more erythrophagocytosis ($P < 0.001$) compared to their respective sham counterparts. Crry$^{+/-}$ infected mice had significantly more erythrophagocytosis compared to wild-type infected animals ($P = 0.027$). (C) Representative H&E stained liver section at 63X magnification from a wild-type (right) and Crry$^{+/-}$ (left) *P. chabaudi*-infected animal. Green arrow indicates a macrophage with internal red cells. H&E stained tissue sections were converted into a digital format using a Nanozoom with NDP.scan software. Image files were viewed, annotated, and exported to .jpg format using NDP.vew software.

Figure 8: Histopathology of the liver and spleen of wild-type and Crry$^{+/-}$ *P. chabaudi* infected mice



Crry$^{+/-}$ red cells are more susceptible to in vitro *erythrophagoyctosis when exposed to malaria*

To verify the *in vivo* erythrophagocytosis data, we tested the susceptibility of red cells from wild-type and Crry$^{+/-}$ animals to be phagocytosed by macrophages *in vitro*. Red cells from infected wild-type and Crry$^{+/-}$ animals were significantly more susceptible to phagocytosis compared to their sham counterparts ($P = 0.004$ and $P < 0.001$ respectively; Figure 9). Furthermore, red cells from infected Crry$^{+/-}$ animals were significantly more susceptible to phagocytosis compared to infected wild-type animals ($P = 0.004$; Figure 9), verifying the *in vivo* data.

Crry$^{-/-}$ RBCs are cleared more rapidly in a P. chabaudi *infection*

To gain a better understanding of the kinetics of clearance of red cells in a malaria infection, we transferred fluorescently labeled wild-type, Crry$^{+/-}$, or Crry$^{-/-}$ RBCs into a C57BL/6 *P. chabaudi* infected mouse. The percent of labeled cells in the peripheral blood were monitored by flow cytometry for 5 days post transfer (Figure 10A). Interestingly, partial Crry deficiency (Crry$^{+/-}$) did not result in faster clearance from the peripheral blood; however, complete Crry deficiency (Crry$^{-/-}$) did result in more rapid clearance (Figure 10). The proportion of labeled cells remaining 5 days post transfer (10 days post-infection) was lowest in animals that received Crry$^{-/-}$ RBCs compared to wild-type and Crry$^{+/-}$ RBCs (Figure 10B).

Figure 9: Crry$^{+/-}$ RBCs are more susceptible to *in vitro* phagocytosis than wild-type RBCs. Red cells obtained from wild-type and Crry$^{+/-}$ sham and *P. chabaudi* infected mice were fluorescently labeled with CFSE and incubated with J774 A.1 macrophages in culture. All external RBCs were lysed and macrophages were detached from the culture wells, fixed, and run through an LSRII flow cytometer to detect internal RBCs. RBCs from *P. chabaudi* infected animals were phagocytosed more than RBCs from sham infected animals ($P = 0.004$ and $P < 0.001$ for wild-type and Crry$^{+/-}$ respectively). RBCs from infected Crry$^{+/-}$ animals were significantly more susceptible to phagocytosis compared to RBCs from infected wild-type animals ($P = 0.004$).

Figure 9: Crry$^{+/-}$ RBCs are more susceptible to *in vitro* phagocytosis than wild-type RBCs

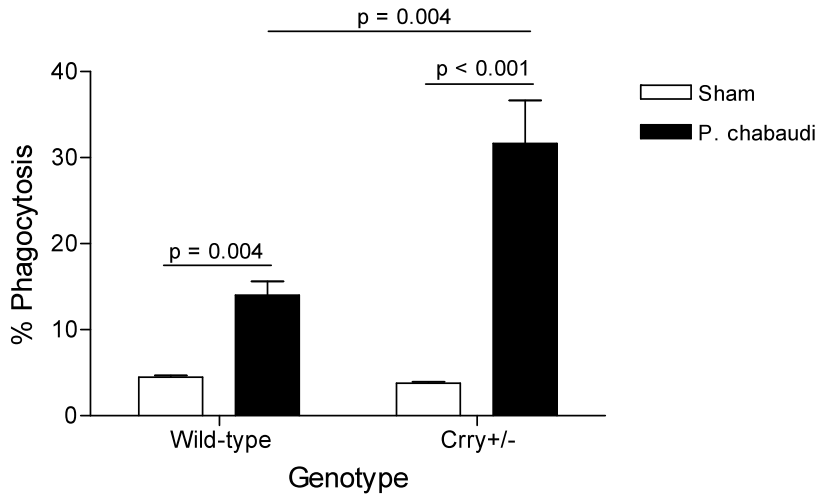
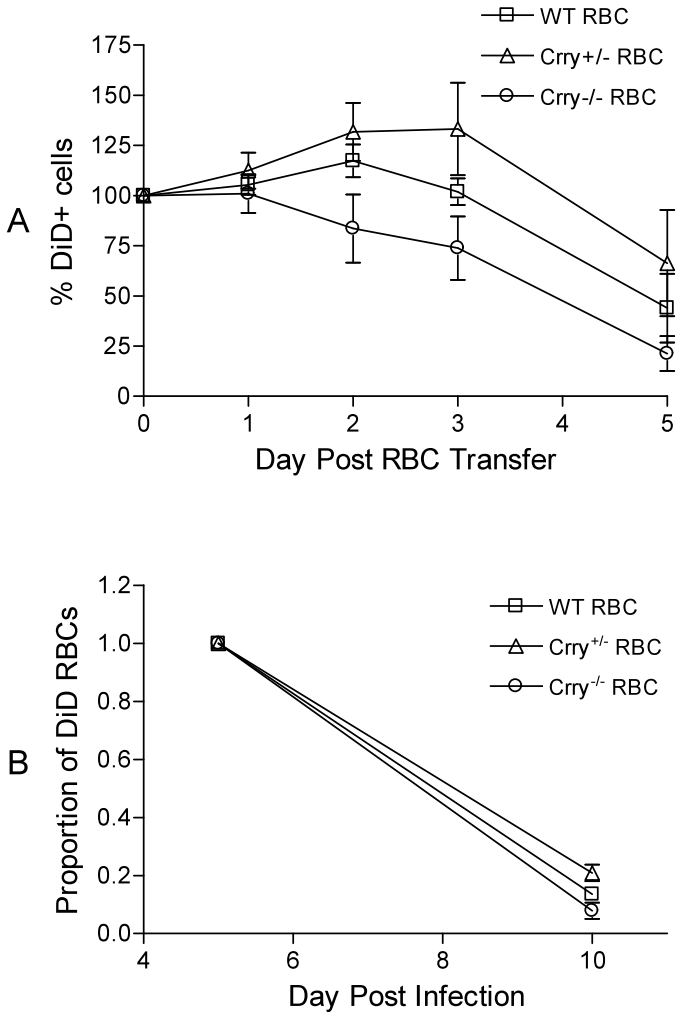


Figure 10: Comparison of wild-type, Crry$^{+/-}$, and Crry$^{-/-}$ RBC survival in a *P. chabaudi* infected mouse. Wild-type, Crry$^{+/-}$, and Crry$^{-/-}$ RBCs were obtained from uninfected animals, labeled *ex vivo* with DiD, and transferred into C57BL/6 mice 5 days post *P. chabaudi* infection. The labeled cells were tracked for 5 days post transfer by flow cytometry. All values are normalized to a baseline value set at 30 minutes post RBC transfer. (A) Kinetics of labeled RBC clearance for full timeframe monitored is shown. (B) Proportion of labeled cells remaining at the final time-point compared to baseline is shown.

Figure 10: Comparison of wild-type, Crry$^{+/-}$, and Crry$^{-/-}$ RBC survival in a *P. chabaudi* infected mouse

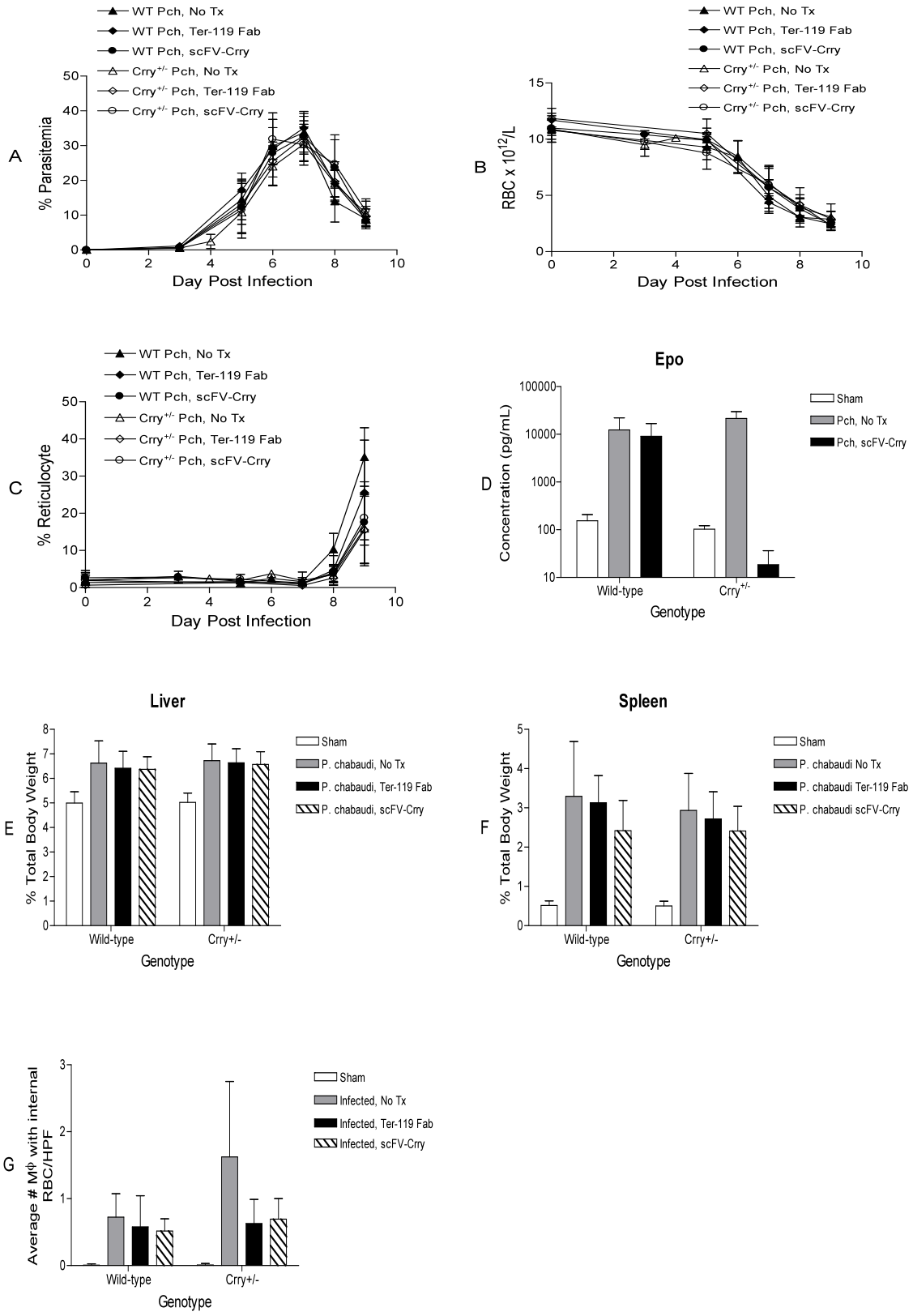


Supplementation of red cell Crry decreases erythrophagocytosis

To further demonstrate that Crry can protect red cells from erythrophagocytosis during malaria infection, we supplemented the amount of Crry on red cells with a single chain FV (scFV) that targets glycophorin and carries Crry[128]. Flow cytometric analysis confirmed the increase in surface Crry for all scFV-Crry treated groups (data not shown). We were able to restore the RBC surface Crry to wild-type levels in the $Crry^{+/-}$ mice. Treating wild-type and $Crry^{+/-}$ mice with scFV-Crry did not affect the level of parasitemia nor the RBC counts, but did decrease the reticulocyte response seen in infected wild-type animals (Figure 11A-C). There was also no difference in liver and spleen weights in scFV-Crry treated animals (Figure 11E-F); however, spleen sizes tended to be smaller in scFV-Crry treated animals compared to untreated animals. Additionally, measurements of plasma Epo concentrations revealed a drastic decline in infected $Crry^{+/-}$ mice that received scFV-Crry compared to untreated mice, while no difference was seen in the infected wild-type mice regardless of treatment status (Figure 11D). Finally, examination of the liver revealed a dramatic decrease in the level of erythrophagocytosis in scFV-Crry treated $Crry^{+/-}$ mice (Figure 11G). The level of erythrophagocytosis was comparable to that of untreated wild-type mice. Additionally, treated wild-type mice showed less erythrophagocytosis in the liver than untreated wild-type mice, although the difference was not significant (Figure 11G). The engorged appearance seen in untreated $Crry^{+/-}$ mice was not observed in scFV-Crry treated mice (data not shown).

Figure 11: Effect of treatment with scFV-Crry on the course of malaria infection. Wild-type (WT) and Crry$^{+/-}$ mice were infected with *P. chabaudi* (Pch) or RPMI/BSA as a sham control; some animals received a single chain FV linked to Crry (scFV-Crry) that added Crry to the surface of red cells or a Ter-119 Fab control. The parasitemia (A), RBC count (B), and reticulocyte levels (C) were monitored throughout the infection. (D) Plasma Epo concentrations ± SD at the point of severe anemia were measured. (E-F) Liver and spleen weights normalized to body weight ± SD at day 9 post infection are shown. (G) Quantitation of erythrophagocytosis in the liver determined from H&E stained tissue sections are shown.

Figure 11: Effect of treatment with scFV-Crry on the course of malaria infection



Discussion

Severe malarial anemia is a complex phenomenon and the pathogenesis remains unclear. Evidence clearly suggests that destruction of uninfected erythrocytes is paramount to the pathophysiology of SMA[22;121;124]. Additionally, we have shown that patients with SMA have an acquired deficiency in the regulatory proteins CR1 and CD55[34;35]. In the presented study, we aimed to understand the role of complement receptor-1 related gene/protein Y (Crry) in the pathogenesis of SMA in mice. Crry is a genetic homologue of human CR1 and a functional homologue of human CD55[108]. Crry was found to be important in protecting red cells from destruction as evidenced by erythrophagocytosis during a *P. chabaudi* infection.

We did not see a difference in the level of anemia between wild-type and $Crry^{+/-}$ animals from RBC counts; however, mice have a well documented extramedullary erythropoietic capacity[129;130], which could be compensating for increased red cell destruction and thus masking differences in the degree of anemia. The differences observed in erythrophagocytois in the liver suggest greater red cell destruction is occurring in Crry deficient animals. Furthermore, supplementing Crry in the deficient animals was able to reduce the increased levels of erythrophagocytosis to levels comparable to wild-type animals. Additionally, increasing Crry levels in wild-type animals appeared to decrease the level of erythrophagocytosis, even though the decline was not statistically significant. Also, Crry supplementation significantly decreased plasma erythropoietin concentrations, suggesting less of a need for stimulation to produce erythroid cells. These data indicate Crry is important in red cell protection during a malaria infection.

Somewhat surprisingly, our RBC transfer study showed that complete lack of Crry, but not partial deficiency, resulted in increased clearance from the peripheral blood. It is possible that an increased volume of labeled $Crry^{+/-}$ cells were transferred compared to either normal or $Crry^{-/-}$ RBCs; however that is unlikely. This presents an interesting idea that there may be a threshold at which complement regulatory proteins are protective. More research needs to be done to explore this further. It is however interesting to speculate what the malarial infection dynamics would be in the context of complete Crry deficiency; however, due to lack of a comparable model, it is not possible to explore this *in vivo.* A Crry knockout mouse has been developed; however, these mice experience accelerated C3 turnover and consequently have reduced alternative pathway activation potential[111].

The exact mechanism of SMA remains to be elucidated. Our data showing increased red cell susceptibility to phagocytosis *in vitro* when exposed to malaria suggests that one mechanism involved is erythrophagocytosis. Additionally, RBCs from patients with SMA have demonstrated increased susceptibility to erythrophagocytosis *in vitro*[35]. We also saw erythrophagocytosis in the liver of *P. chabaudi* infected animals, which has been previously reported in the literature[37], as well as in the livers and spleens of patients who have died from *P. falciparum* malaria[36]. The increased susceptibility of $Crry^{+/-}$ red cells to phagocytosis suggests modifications to the red cells are a contributory factor to SMA. Since the role of Crry is to protect host cells from complement attack, perhaps there is increased C3b deposition on the deficient RBCs, which has been seen in humans[32]. Modification could also be from the parasite by deposition of parasite proteins on the surface of uninfected red cells, which could then be targeted by antibodies and

cleared by phagocytes. Studies have found *Plasmodium* rhoptry proteins on the surface of uninfected red cells and increased destruction by antibody-mediated clearance[38;39;131;132]. Further studies need to be conducted to investigate the contribution of C3 and immune complexes in the pathogenesis of SMA.

In addition to seeing increased red cell destruction in animals with partial Crry deficiency, we saw a decline in surface Crry levels on uninfected RBCs obtained from infected animals. This decline may actually be greater than it appears due to the fact that there is an influx of reticulocytes, which have a higher level of Crry than mature red cells, that could be skewing the overall expression levels. Perhaps this decline in Crry is facilitating the increased phagocytosis observed in the liver, which could be mediated by complement. Further experiments need to be conducted to investigate the role complement has, if any, in this model. However, the decline in surface Crry corresponds with the acquired deficiency in CR1 and CD55 observed in children with SMA[34;35]. Extrapolating these data to human *P. falciparum* infection suggests deficiencies in regulatory proteins make red cells more susceptible to destruction, presumably by erythrophagocytosis, which leads to more severe anemia. This presents a need to protect existing red cells during treatment of malaria, perhaps by facilitating an increase in the regulatory proteins on the surface of red cells.

While using a mouse model of malaria infection is beneficial because we can directly investigate immunopathogenic mechanisms with knockout animals, there are limitations. One of the limitations to using mouse models is that they develop high levels of parasitemia[117], which is not seen in patients with SMA[133]. The hyperparasitemia in mice infected with *P. chabaudi* may mask apparent differences in the level of anemia

because up to 40% of the red cells are being destroyed directly by the parasite. However, the degree of anemia in *P. chabaudi*-infected animals is still out of proportion to the level of parasitemia, which would indicate that other potential immune responses leading to SMA are still occurring. Our findings of increased red cell destruction in the liver of animals with partial Crry deficiency may show enhanced severity of anemia at the level of the peripheral blood in a mouse model of low parasite burden. Another limitation to this model is the inability to assess the effect of subsequent re-infection with the same parasite due to the development of sterile immunity. SMA is more often found in areas of endemic transmission[8] where children suffer repeated infections[134]. We have to consider this caveat when examining immune responses that could be contributing to SMA.

In summary, we have shown Crry to be important in protecting red cells from erythrophagocytosis during a malaria infection. This suggests complement regulatory proteins are important in the pathogenesis of severe anemia and that erythrophagocytosis is a potential mechanism in the development of SMA. Further research is needed to investigate the role complement could have in this system.

Chapter 4

Role for C3 in the pathogenesis of SMA

Introduction

As previously mentioned, complement is a component of the innate immune system and is important in protecting a host during an infection[71]; however, dysregulation of this system can have devastating effects. All complement activation pathways converge at the point of C3 activation[73]; therefore, it is not out of context to believe C3 could be involved in the pathogenesis of disease associated with an invading pathogen. Malaria activates both the classical and alternative pathways during a complicated infection[135] and a role for complement activation in the pathogenesis of malarial anemia was suggested by correlations between positive DCT and malaria patients with anemia[31;136;137]. Furthermore, patients with SMA have been shown to have increased deposition of C3b on their red cells[32;33].

To investigate complement's contribution to the pathogenesis of SMA we studied the role of C3 in the destruction of red cells during a *Plasmodium chabaudi* infection in mice. Other research, presented in chapter 3, showed that the complement regulatory protein Crry was important in protecting red cells during a malaria infection. Given the primary role for complement regulatory proteins is to provide protection to host cells from autologous complement activation, we wanted to ascertain whether the complement component C3 was also important in the development of SMA. In the study presented here, we utilized *P. chabaudi*-infected wild-type and C3 deficient animals to determine if C3 was important in the destruction of red cells during a malaria infection.

Results

C3 deficiency results in less severe anemia during P. chabaudi *infection*

To characterize the severity of disease, basic parameters of the blood were monitored during a *P. chabaudi* infection. Wild-type and $C3^{-/-}$ *P. chabaudi*-infected animals experienced similar infection kinetics except for their RBC counts. There was no difference in the parasitemia levels between wild-type and $C3^{-/-}$ animals (Figure 1A) and both genotypes experienced an increase in their reticulocyte counts to the same degree (Figure 12C). However, $C3^{-/-}$ *P. chabaudi*-infected animals had a significantly higher RBC count at days 7 and 8 post-infection compared to wild-type infected animals (Figure 12B).

Clearance patterns of Crry deficient RBCs differs between wild-type and $C3^{-/-}$ P. chabaudi-*infected mice*

In an attempt to elucidate potential mechanism(s) involved in RBC clearance during a malaria infection, we transferred fluorescently labeled wild-type, $Crry^{+/-}$, and $Crry^{-/-}$ RBCs into wild-type and $C3^{-/-}$ *P. chabaudi*-infected animals. The RBC transfer did not affect the development of parasitemia (Figure 13A) and all animals still developed anemia (Figure 13B). However, a difference was seen in the spleen and liver weights among the various groups (Figure 13C and D). Animals that received $Crry^{-/-}$ RBCs had heavier livers and spleens compared to animals that received wild-type or $Crry^{+/-}$ RBCs. When comparing wild-type and $C3^{-/-}$ *P. chabaudi* infected animals that were infused with $Crry^{-/-}$ RBCs, there is a significant decrease in liver ($P = 0.048$) and spleen ($P = 0.009$) weights in the $C3^{-/-}$ animals.

Figure 12: C3 deficiency results in less severe anemia during a malaria infection. C57BL/6 wild-type and $C3^{-/-}$ mice were infected with 10^6 *P. chabaudi* or were sham infected with RPMI/BSA and blood kinetics were monitored throughout the course of infection (each group n=10). (A) Average parasitemia levels are shown. (B) Average RBC counts as a percentage of baseline values are shown. $C3^{-/-}$ *P. chabaudi*-infected animals have significantly higher counts at days 7 and 8 post-infection compared to wild-type *P. chabaudi*-infected animals (* $P = 0.014$ and ** $P < 0.001$ respectively). (C) Average reticulocyte levels are shown.

Figure 12: C3 deficiency results in less severe anemia during a malaria infection

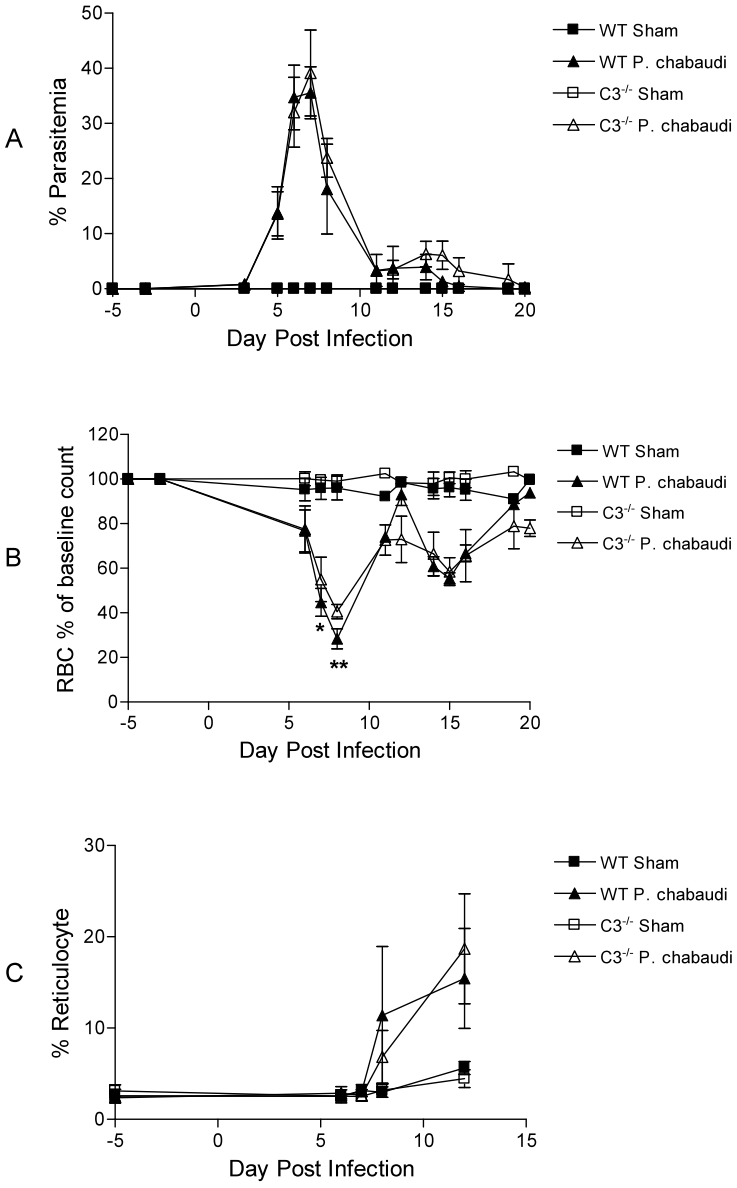
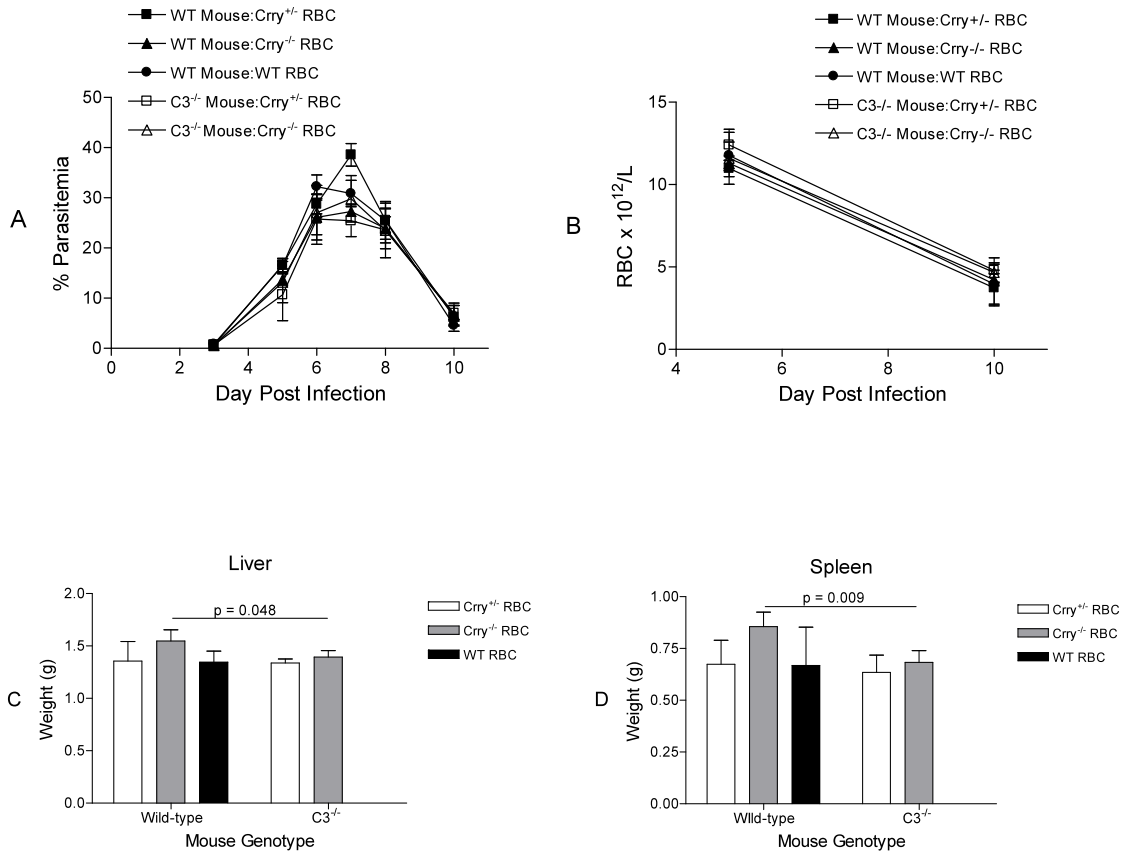


Figure 13: Increased organ pathology from Crry$^{-/-}$ RBC transfer is decreased in absence of C3. Wild-type and C3$^{-/-}$ *P. chabaudi*-infected animals had DiD labeled wild-type (WT), Crry$^{+/-}$, or Crry$^{-/-}$ RBCs transfused via the retro-orbital plexus at 5-days post infection. (A) Average parasitemia levels are shown. (B) Average RBC counts prior to RBC transfer and just prior to euthanasia are graphed. (C) Average liver weight in grams ± SD are shown. C3$^{-/-}$ mice that received Crry$^{-/-}$ RBCs had significantly (P = 0.048) smaller livers compared to wild-type animals that received the same type of RBCs. (D) Average spleen weight in grams ± SD are shown. C3$^{-/-}$ mice that received Crry$^{-/-}$ RBCs had significantly (p = 0.009) smaller spleens compared to wild-type animals that received the same type of RBCs.

Figure 13: Increased organ pathology from Crry$^{-/-}$ RBC transfer is decreased in the absence of C3



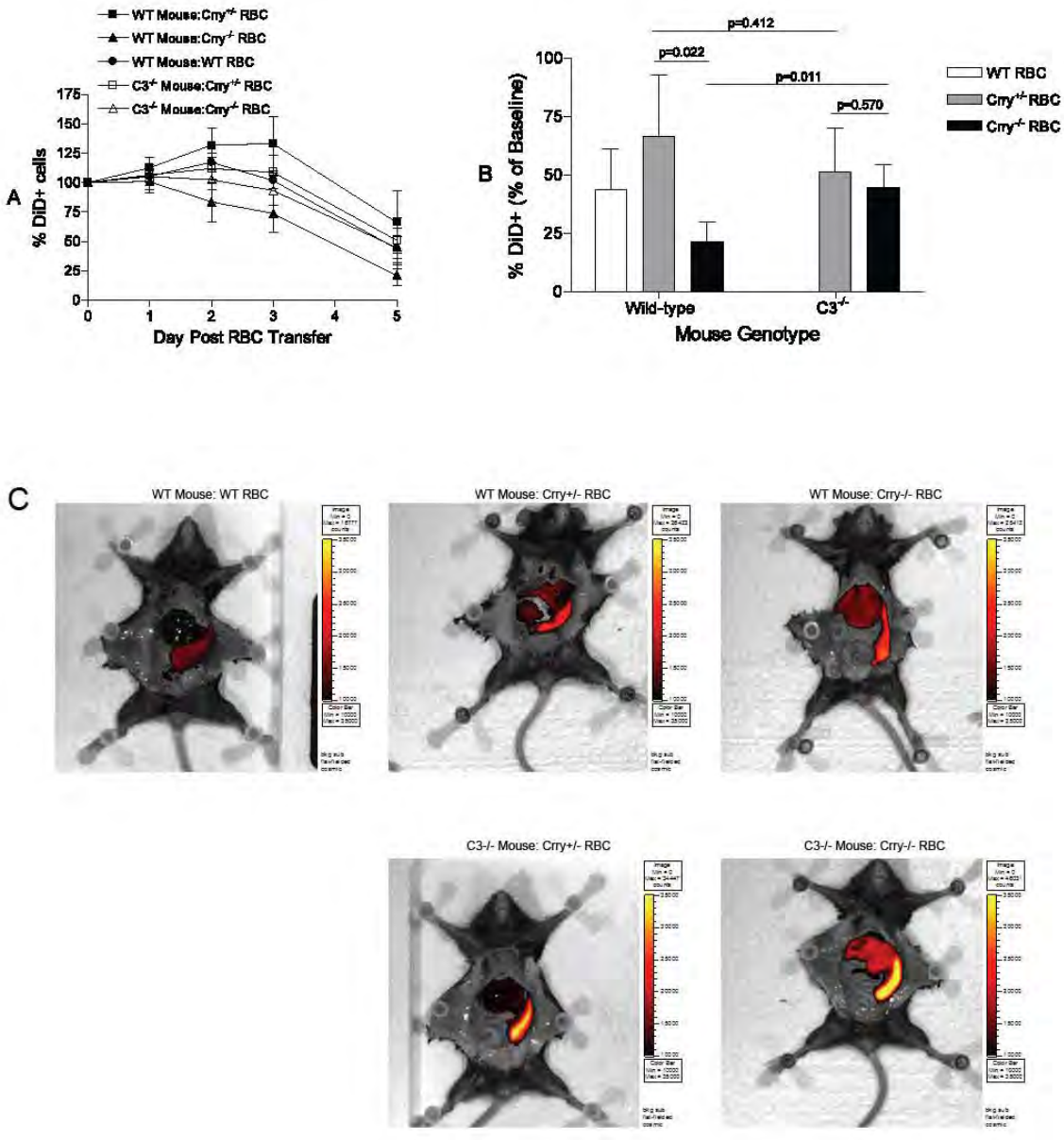
In addition to examining the infection kinetics and organ pathology, we monitored the clearance pattern of the labeled RBCs from the peripheral blood by flow cytometry. Figure 14A shows the kinetics of clearance over the timeframe in which the labeled RBCs were monitored. In a wild-type animal, $Crry^{-/-}$ RBCs were cleared more rapidly from the periphery than either wild-type or $Crry^{+/-}$ RBCs. In the absence of C3 the difference in clearance rate between $Crry^{-/-}$ and $Crry^{+/-}$ RBCs was not seen. At 5 days post RBC transfer there were significantly fewer remaining $Crry^{-/-}$ RBCs in the wild-type animals compared to $Crry^{+/-}$ RBCs ($P = 0.022$) (Figure 14B). However, in the $C3^{-/-}$ animals there was no difference in the amount of $Crry^{+/-}$ and $Crry^{-/-}$ RBCs. In fact, there were significantly more $Crry^{-/-}$ RBCs remaining in the $C3^{-/-}$ animals compared to wild-type ($P = 0.011$) (Figure 14B). The differences in RBCs remaining in wild-type versus $C3^{-/-}$ animals was not seen for $Crry^{+/-}$ RBCs, suggesting there is a threshold at which Crry protects RBCs from C3 during *P. chabaudi* infection.

$C3^{-/-}$ P. chabaudi-infected animals sequester Crry deficient RBCs to a greater extent than wild-type animals

In order to understand where the RBCs went once they were removed from the peripheral blood, we used *in vivo* imaging technology (IVIS) to view the organs of animals that received fluorescently labeled RBCs. $Crry^{+/-}$ RBCs were detected at above background levels in the spleen and liver of wild-type animals, and in the spleen of $C3^{-/-}$ animals. $Crry^{-/-}$ RBCs were detected in the spleen and liver of both wild-type and $C3^{-/-}$ animals (Figure 14C). For both $Crry^{+/-}$ and $Crry^{-/-}$ RBCs, the intensity of fluorescence

Figure 14: Clearance of Crry deficient RBCs is different between wild-type and C3$^{-/-}$ *P. chabaudi*-infected animals. Wild-type and C3$^{-/-}$ *P. chabaudi*-infected animals received DiD labeled wild-type, Crry$^{+/-}$, or Crry$^{-/-}$ RBCs at day 5 post-infection. Labeled RBCs were monitored in the peripheral blood by flow cytometry and presented as average percent of DiD cells as a percent of baseline, which was set by a 30 minute post-transfer time-point. At day 10 post-infection (5 days post-RBC transfer) animals were euthanized and their abdomens were surgically opened and visualized with a Xenogen IVIS 50 Imaging System using Living Image software. (A) Kinetics of clearance as monitored for 5 days post-RBC transfer are shown. (B) Remaining RBCs presented as average percent of DiD+ cells as a percent of baseline ± SD for day 5 post-RBC transfer (day 10 post-infection) are shown. (C) Representative fluorescent images of mice that received DiD labeled RBCs are shown. A Cy5.5 filter was used with a 5 second exposure. Dark red indicates less intensity, while yellow indicates more intense signal from the DiD dye. Crry$^{+/-}$ and Crry$^{-/-}$ RBCs trafficked to the spleen and liver in both wild-type and C3$^{-/-}$ animals; however, there is greater sequestration seen in C3$^{-/-}$ animals.

Figure 14: Clearance of Crry deficient RBCs differs between wild-type and C3$^{-/-}$ animals



was greater in the $C3^{-/-}$ animals, suggesting that RBCs in the C3 deficient environment were being sequestered to a greater extent in the spleen and liver. These findings also suggest a difference in the mechanism(s) of RBC clearance when C3 is present.

Discussion

Severe malarial anemia is complex and the mechanisms of pathogenesis are not clearly understood. Our research has shown a role for complement regulatory proteins in the protection of red cells during a malaria infection (chapter 3), which led to an obvious question regarding the role of complement in the pathogenesis of anemia. In the presented study, we aimed to understand the role of C3 in the development of SMA. C3 is the most abundant serum complement protein and the point of convergence of all three activation pathways[71]. The data presented here indicate that C3 has a role in the pathogenesis of SMA; however the mechanistic connection may not be as direct as we initially believed. C3 deficient animals infected with *P. chabaudi* had significantly higher RBC counts compared to wild-type animals at the nadir of their anemia, suggesting C3 is increasing the severity of anemia.

If C3 is increasing the severity of anemia, what is the mechanism involved? Activated C3 yields 2 products, C3a and C3b, which can elicit different effects[73]. C3a is an anaphylatoxin involved in inflammation and chemotaxis/activation of phagocytes[82;83], while C3b continues activation of the complement cascade and can opsonize cells for phagocytosis[86;97]. Our work with complement regulatory proteins suggests erythrophagocytosis in the liver is involved in the pathogenesis of SMA. Unfortunately, at this point we have not fully investigated this avenue with the C3 deficient animals.

Pathology of the spleen and liver needs to be examined, including a detailed analysis of the histopathology. If erythrophagocytosis is involved, I would expect to see significantly less erythrophagocytosis in the C3 deficient animals. Additionally, I would expect the spleens from C3 deficient animals to be smaller and show less evidence of extramedullary erythropoiesis.

Since our research focuses on the role of complement and complement regulatory proteins in the pathogenesis of SMA, we investigated the interplay of the regulatory protein Crry and C3 to see if there was a relationship in the development of anemia. Crry is a rodent specific complement regulatory protein that has its regulatory effect at the level of C3 activation[108]. We saw some clearance of all infused red cells indicating that to some degree all the cells were damaged, likely from the *ex vivo* labeling process. However, there was increased clearance of $Crry^{-/-}$ RBCs compared to wild-type and $Crry^{+/-}$ RBCs in wild-type *P. chabaudi*-infected animals. The increased clearance of Crry deficient RBCs in wild-type animals was ameliorated when C3 was absent, suggesting that C3 is important in red cell clearance during a malaria infection. The complete deficiency in Crry could be allowing a greater deposition of C3b on the red cell surface and subsequent clearance by phagocytes. Increased C3b deposition on red cells has been observed in patients with SMA[32;33].

Interestingly, we saw a difference in the sequestration of $Crry^{+/-}$ and $Crry^{-/-}$ red cells between wild-type and C3 deficient infected animals. The RBCs trafficked to the spleen and liver in both animal types; however, there was greater sequestration in the C3 deficient animals. This seems counterintuitive given that clearance from the peripheral blood was less in the absence of C3. However, these data indicate a different process is

occurring when C3 is absent versus when it is present. One possibility is that in the absence of C3, red cells are reversibly sequestered in the spleen and liver. On the other hand, when C3 is present the red cells may be actively destroyed; this could occur via phagocyotosis due to opsonization with C3b. These observations all need to be confirmed with more animals and further tracking experiments. Examination of the spleen and liver utilizing fluorescent microscopy could help ascertain what is occurring to the cells that are trafficking to those organs. Additionally, examination of the liver for erythrophagocytosis could help answer whether there is greater RBC destruction in wild-type versus C3 deficient animals.

These studies utilize the same animal model as discussed in chapter 3 and are therefore afflicted by the same limitations: hyperparasitemia and inability to assess subsequent re-infections with the same parasite. High level parasitemia can mask potential differences in anemia; although a difference was observed between wild-type and C3 deficient animals. This difference in anemia may be even greater than what was measured, which could be brought to light in a model with a low parasite burden. Immune complexes are involved in the classical pathway of complement activation[71] and have been associated with SMA[34]. The current model does not adequately address this issue because of the inability to evaluate subsequent re-infections. To fully investigate the role of complement and complement regulatory proteins in the context of a malaria infection and the development of SMA, a model that is more representative of *P. falciparum* infection needs to be available. This rationale prompted us to develop a model of malarial anemia with a low parasite burden, which is discussed in chapter 5.

Chapter 5

Sequential Plasmodium chabaudi-Plasmodium berghei *infection of C57BL/6 mice provides novel model for studying severe malarial anemia*

Based on manuscript submitted for publication review to Blood Journal

Abstract

We describe here a novel model of severe malarial anemia (SMA) in mice. C57BL/6 mice infected sequentially with *Plasmodium chabaudi* AS followed, after recovery, by infection with *P. berghei* ANKA developed severe anemia with relatively low parasitemia within 7 days post-infection. This was followed by a second phase of uncontrolled parasitemia, reticulocytosis, more profound anemia, and death 14-21 days after infection with *P. berghei*. By contrast, naïve animals infected with *P. berghei* died of CM 6-7 days after infection. Compared to *P. berghei*-infected mice, *P. chabaudi/P. berghei*-infected animals had high IL-10/TNF-α and IL-12/IFN-γ ratios. In addition, model animals showed higher antibody levels against both *P. berghei* and *P. chabaudi* than animals infected singly with either species. Treatment with chloroquine on day 5 post-infection with *P. berghei,* when the parasitemia was 3-4%, failed to prevent the development of anemia, suggesting that the anemia was not directly related to destruction of red cells by the parasite. In addition, red cell tracking experiments showed accelerated removal of *ex vivo* labeled RBCs from the circulation. This is a highly reproducible and

practical model of SMA that will be useful in dissecting the pathogenesis of SMA, as well as understanding the immune responses associated with this complication.

Introduction

Plasmodium falciparum is an intracellular parasite of humans responsible for 1-2 million deaths per year. Severe malarial anemia (SMA) due to *Plasmodium falciparum* claims the lives of thousands of children in sub-Saharan Africa every day. The pathogenesis of this anemia is complex and not well understood. There is evidence supporting a role for bone marrow suppression [24;138], as well as evidence to suggest that uninfected red blood cells are destroyed at an accelerated rate in a manner independent of the level of parasitemia[59;124;125]. Data obtained from patients infected with *P. falciparum* to treat neurosyphilis has been used in mathematical modeling to show that an average of 8.5 uninfected red cells are destroyed for every parasitized red cell[22]. A prospective study in a Karen community on the western border of Thailand showed that in anemia caused by *P. falciparum,* the proportion of red cell mass loss attributable to the parasite was 7.9% of the total red cell loss[23]. Additionally, patients treated for *P. falciparum* malaria continued to experience red cell destruction after treatment, indicating that the parasite is not directly responsible for the majority of the destruction of red cells that is resulting in anemia[126].

The study of host and parasite factors that contribute to the pathogenesis of SMA has been hampered by the lack of an inexpensive and reproducible animal model that is relevant to the clinical picture seen with *P. falciparum* infection. Although there are currently multiple rodent models available, all differ significantly from the clinical picture of severe anemia seen with *P. falciparum*. The rodent parasites most commonly

used to study anemia are *P. chabaudi, P. berghei, P. vinckei,* and *P. yoelii*[28]. Specific components of the immune response to these parasites have been studied with the use of inbred strains of mice. However, the virulence of each of these rodent *Plasmodia* species varies depending on the strain of mouse used. *P. berghei* and *P. vinckei* are lethal in all strains of mice, while *P. chabaudi* and *P. yoelii* are only lethal in some strains[115;117]. *P. berghei* ANKA causes death from cerebral complications in C57BL/6 and CBA mice before severe anemia develops and is lethal in CD-1 mice with the development of anemia[114;118]. Finally, *P. chabaudi* AS infection causes severe anemia with hyperparasitemia of 30 to 40%, differing in lethality depending upon the strain of mouse used[119;120].

Recently, Evans *et al*.[121] described a model of SMA by *P. berghei* ANKA infections in semi-immune BALB/c mice and naïve Wistar rats. These animals developed severe anemia in the presence of a low parasite burden, which is similar to what is seen in human *P. falciparum* infection. They also demonstrated an accelerated destruction of uninfected red cells, which has been reported in humans infected with *P. falciparum*[22]. While this model does represent significant improvement over previous models, its biggest limitation is the long preparative time (up to six months) required to establish partial immunity with repeated cycles of infection and drug cure. The timing of the anemia is also unpredictable, making it difficult to plan experiments.

In addition to rodent models, there are non-human primate models of malarial infection. Semi-immune *Aotus* monkeys infected with *P. falciparum* have been used to study severe malarial anemia[122]. While the use of primates is advantageous due to their similarity to humans, their short supply and cost make this approach less attractive.

Based on the above there is a critical need to develop a model of SMA that is inexpensive, highly reproducible, and relevant to human malarial infections. Therefore, we sought to develop this model in C57BL/6 mice and report here its initial characterization.

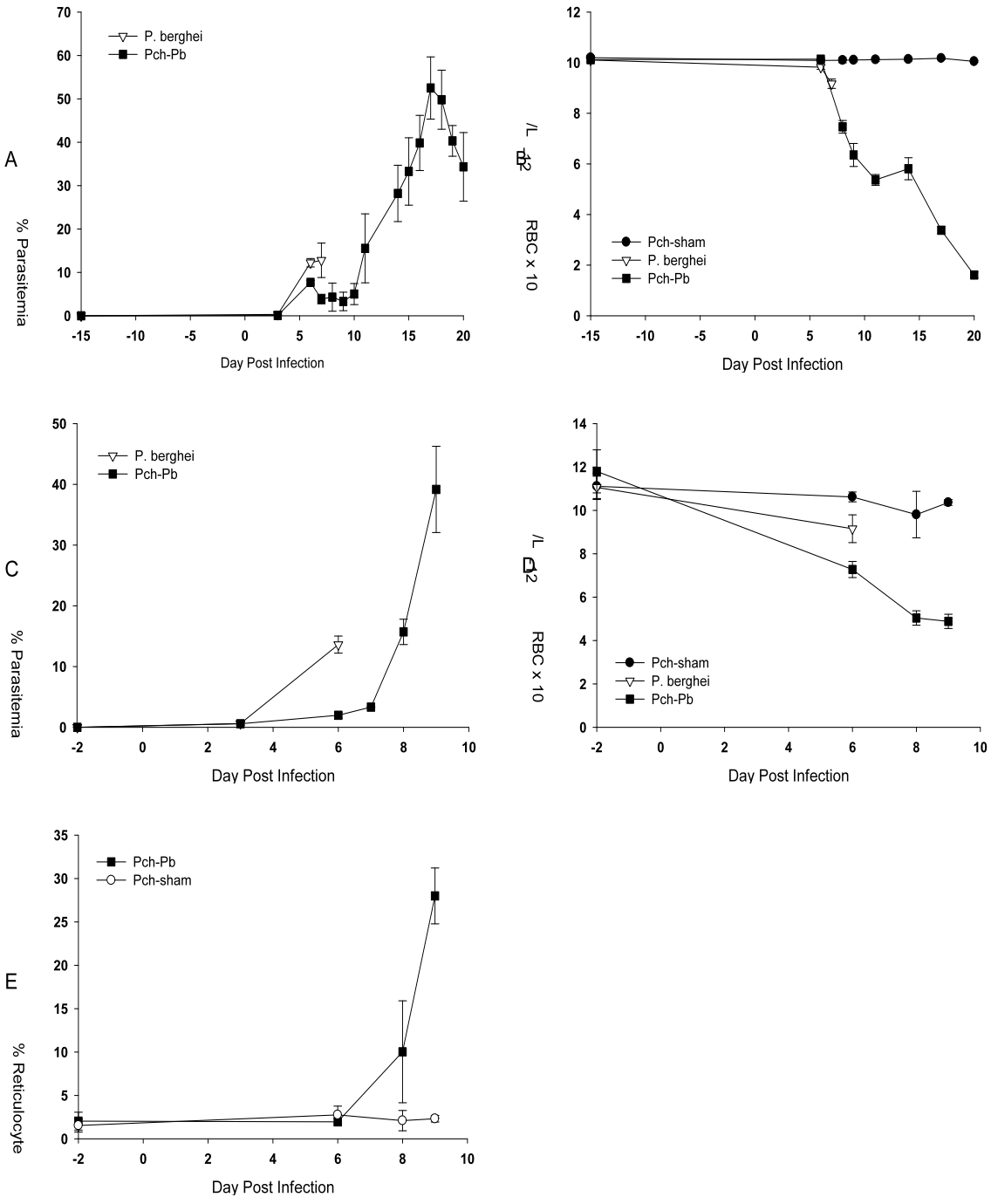
Results

P. chabaudi/P. berghei *infection leads to severe anemia with low parasitemia*

Our approach was based on the premise that some level of pre-immunity was required for the development of SMA. With this in mind, we tested a number of strategies to confer partial immunity to mice which included injecting mice with IRBCs containing parasites killed by different methods such as freeze-thawing, heating, and irradiation followed by homologous challenge. All of these induced solid homologous sterile immunity. Therefore, we decided to test *P. chabaudi* infection followed by heterologous *P. berghei* challenge. C57BL/6 mice infected with *P. berghei* ANKA following recovery from a *P. chabaudi* AS infection developed SMA with an accompanying low parasitemia for the first 10 days of infection (Figure 15A). The mice exhibited a decline in their RBC counts that was disproportionate to the level of parasitemia (Figure 15B). The mice eventually died from progressively rising parasitemia and worsening anemia around day 20. On the other hand, naïve C57BL/6 mice infected with *P. berghei*, developed a higher parasitemia without a dramatic decline in RBC count and died by day 7 post-infection from cerebral complications (Figure 15A and B). Mice that were previously infected with *P. chabaudi* and were then sham infected did not develop parasitemia or anemia.

Figure 15: ***P. chabaudi/P. berghei* infection yields anemia with low parasitemia.** C57BL/6 mice were infected with 10^6 *P. berghei* following recovery from a *P. chabaudi* infection (Pch-Pb) or in a naïve state (*P. berghei*) or were sham infected with RPMI/BSA (Pch-sham). Average parasitemia levels (A) and RBC counts (B) were monitored throughout the course of infection until the animals died (each group n=5). (C-E) Average infection kinetics of a representative abbreviated infection are shown. *P. berghei* (n=5) mice died 6 days post-infection, while *P. chabaudi*/Sham (n=4) and *P. chabaudi/P. berghei* (n=4) mice were euthanized 9 days post-infection. Parasitemia (C), RBC counts (D), and reticulocyte levels (E) were monitored.

Figure 15: *P. chabaudi/P. berghei* infection yields anemia with low parasitemia



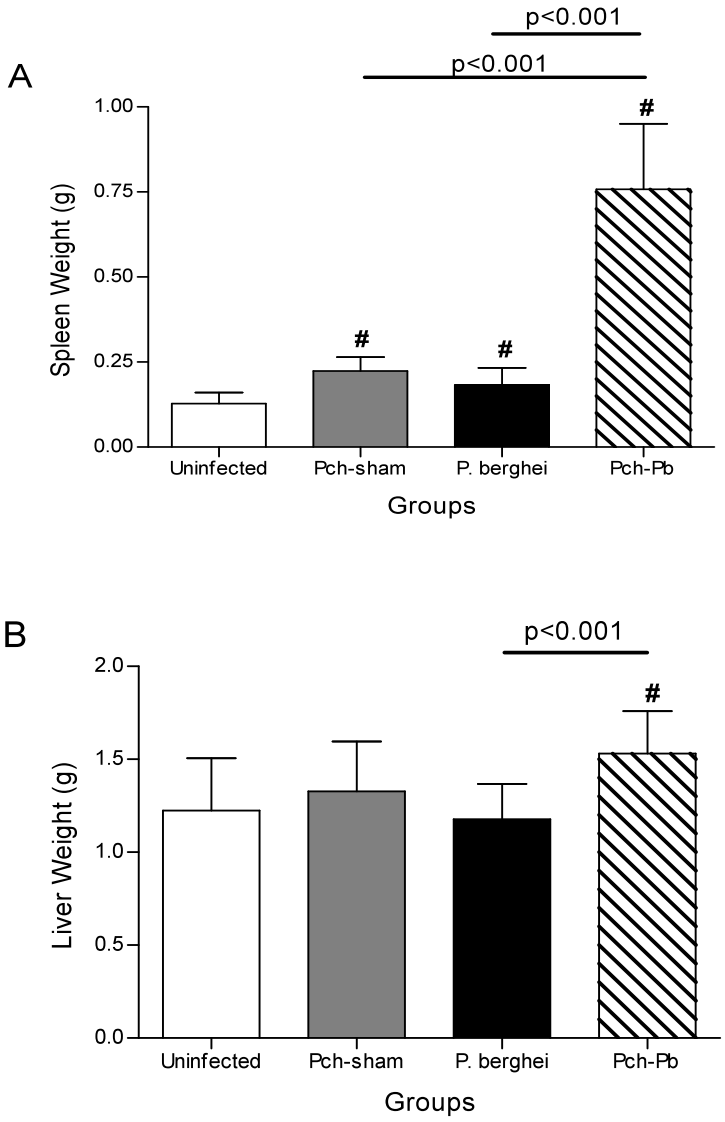
Since we were interested in studying the mechanism of severe anemia during the period of low parasitemia, subsequent abbreviated infections were carried out where mice were euthanized approximately 9-10 days post-infection. In these shorter experiments, mice exhibited the characteristic disproportionate decline in their RBC counts with relatively low level parasitemia (Figure 15C and D). Interestingly, the rise in parasitemia always coincided with the rise in the reticulocyte count (Figure 15E). Microscopic evaluation of these smears during this second phase of parasitemia revealed that the majority of infected RBCs were reticulocytes, reflecting the preference of *P. berghei* for reticulocytes[139;140].

Liver and spleen pathology in the P. chabaudi/P. berghei *mouse model*

To characterize the pathology in the liver and spleen, we harvested these organs from the mice at day 9-10 post *P. berghei* infection in *P. chabaudi/P. berghei*-infected animals or just prior to death for the *P. berghei*-infected animals. Both the liver and spleen were enlarged in *P. chabaudi/P. berghei*-infected mice compared to uninfected and *P. berghei*-infected mice (Figure 16). The spleens from *P. chabaudi/P. berghei*-infected animals were significantly ($P<0.001$) larger and heavier than those from uninfected, *P. chabaudi*/sham-infected, and *P. berghei*-infected animals (Figure 16A). The spleens of *P. berghei*-infected mice were heavier than those of uninfected mice, but not more than those of *P. chabaudi*/sham-infected animals (Figure 16A). Livers from *P. chabaudi/P. berghei* infected mice were heavier than those from *P. chabaudi*/sham-infected, *P. berghei*-infected, and uninfected animals, but the difference was only statistically significant for the latter two groups (Figure 16B). Compared to *P. berghei*-

Figure 16: Infected animals have enlarged liver and spleen. (A) Spleen weight in grams ± SD are shown. Uninfected are significantly smaller than all other groups. *P. chabaudi/P. berghei* are significantly larger than *P. chabaudi*/sham and *P. berghei*. (B) Liver weight in grams ± SD are shown. *P. chabaudi/P. berghei* are significantly larger than Uninfected and *P. berghei*. Uninfected (n=9), Pch-sham (n=8), *P. berghei* (n=11), Pch-Pb (n=9) # indicates a P value of < 0.05 (compared to Uninfected)

Figure 16: Infected animals have enlarged liver and spleen



infected animals, histological evaluation of the spleen in *P. chabaudi*/sham-infected mice showed enlargement of the lymphoid follicles and infiltration of the red pulp by erythropoietic precursors as shown by expansion of TER-119^{+}/Ki67^{+} cells by flow cytometry (Figure 17). Spleens from *P. chabaudi/P. berghei*-infected mice showed disorganized expansion of the white pulp follicles and much greater infiltration by erythropoietic precursors in the red pulp than *P. chabaudi*/sham-infected animals (Figures 17 and 18A). These findings are consistent with the finding of SMA in the *P. chabaudi/P. berghei*-infected animals.

Microscopic examination of the liver showed that hemozoin pigment was present within macrophages in all animals, but more markedly so in *P. chabaudi/P. berghei*-infected animals. Liver sections from *P. chabaudi/P. berghei*-infected animals revealed islands of erythropoietic precursors that were absent in all other groups (Figure 18B).

Figure 17. Ki-67 and Ter-119 characterization of mouse spleens. Spleen cells post RBC lysis were stained with FITC-labeled anti-Ki-67 and PE-labeled rat anti-mouse Ter-119. The Ter-119 population was determined amongst all cells (white bars) and Ter-119^{+} cells were gated and further examined for their Ki-67 characteristics (black bars).

Figure 17: Ki-67 and Ter-119 characterization of mouse spleens

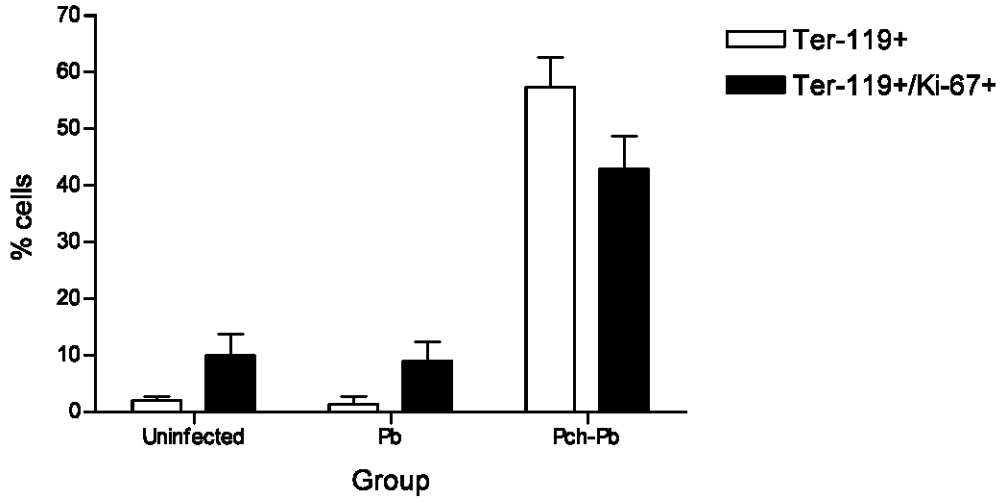
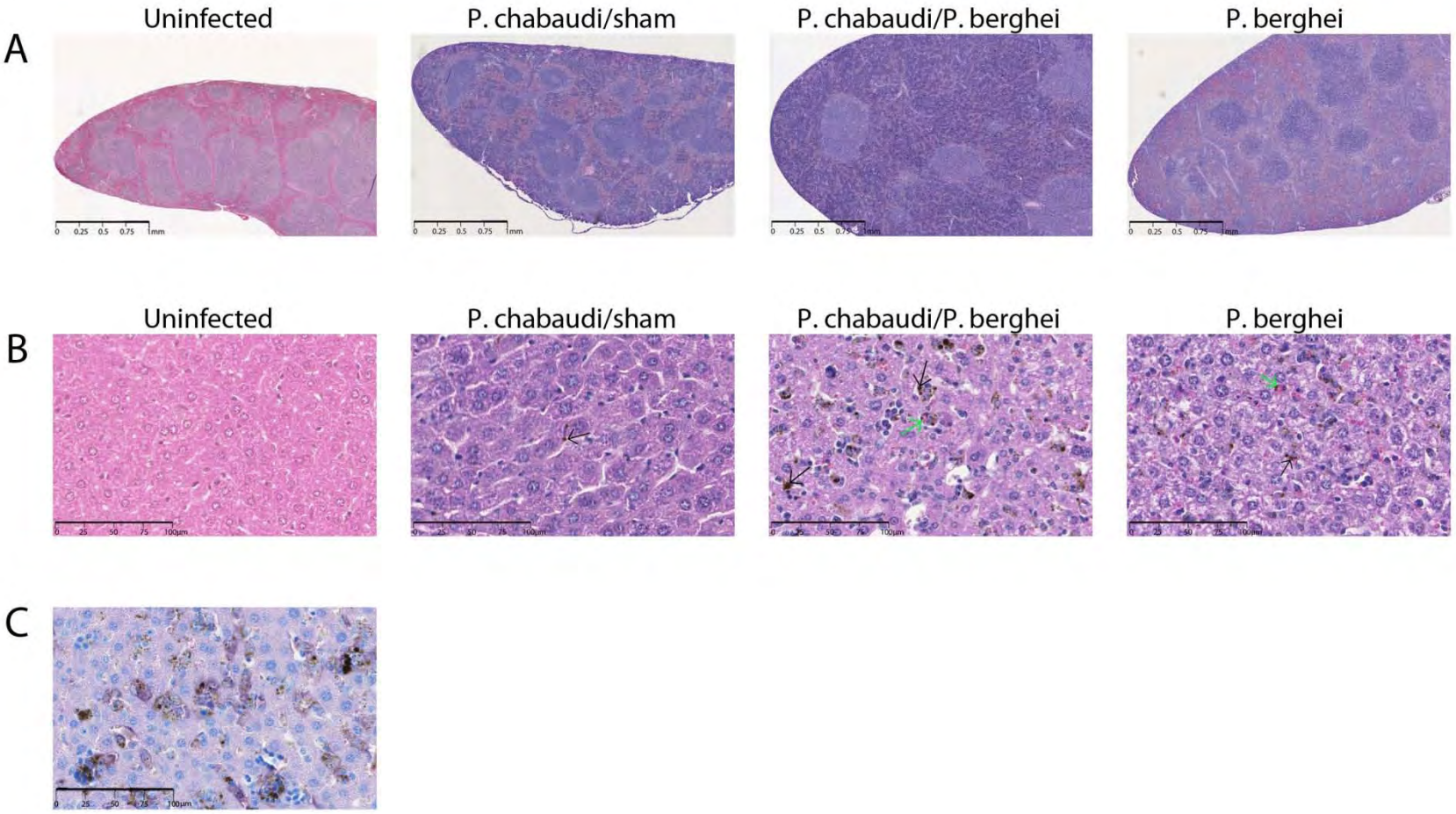


Figure 18: **Spleen and liver pathology.** H&E stained tissue sections were converted into a digital format using a Nanozoom with NDP.scan software. Image files were viewed, annotated, and exported to .jpg format using NDP.view software. (A) Representative H&E stained spleen sections are shown. Images are at 5X magnification. (B) Representative H&E stained liver sections are shown. Images are at 63X magnification. Hemazoin pigment is indicated by black arrows and macrophages with internal RBCs are indicated with green arrows. (C) F4/80 immunohistochemical staining of *P. chabaudi/P. berghei*-infected liver is shown. Deep purple is F4/80 positive staining and red cells can be seen in pink. DAB + Ni was used as the developing chromagen with Giemsa as a counterstain.

Figure 18: Spleen and liver pathology



Increased erythrophagocytosis in P. chabaudi/P. berghei-*infected animals*

To evaluate potential mechanisms of uninfected RBC destruction in the model, liver sections were examined for evidence of erythrophagocytosis. All infected animals had significantly more erythrophagocytosis than sham animals, with *P. chabaudi/P. berghei*-infected animals showing the highest levels (Table 2). When *P chabaudi/P. berghei*-infected animals were treated with cobra venom factor to deplete C3, the level of erythrophagocytosis was significantly decreased (P=0.002) compared to untreated animals (Table 2). The findings in the H&E liver sections were verified by immunohistochemical staining for the macrophage marker F4/80 (Table 2); an example of the staining is seen in Figure 18C. An *in vitro* phagocytosis assay further demonstrated that RBCs from *P. chabaudi/P. berghei*-infected animals were more susceptible to phagocytosis by macrophages compared to either *P. chabaudi*/sham or *P. berghei*-infected animals (Table 2).

Table 2. Erythrophagocytosis in the liver and *in vitro*

	P. chabaudi-Sham	*P. berghei*	*P. chabaudi*-*P. berghei*	*P. chabaudi*-*P. berghei* CVF	P value for comparison between columns 1 and3	P value for comparison between columns 2 and 3	P value for comparison between columns 3 and 4
Average # liver Mφ with internal RBC/HPF	0	0.44	0.60	0.25	0.001	0.200	0.002
% liver erythrophagocytosis	0	17.15	27.43	11.44	<0.001	0.011	0.004
% phagocytosis in J774A.1 Mφ	1.02	3.65	29.45	NA	<0.001	<0.001	NA

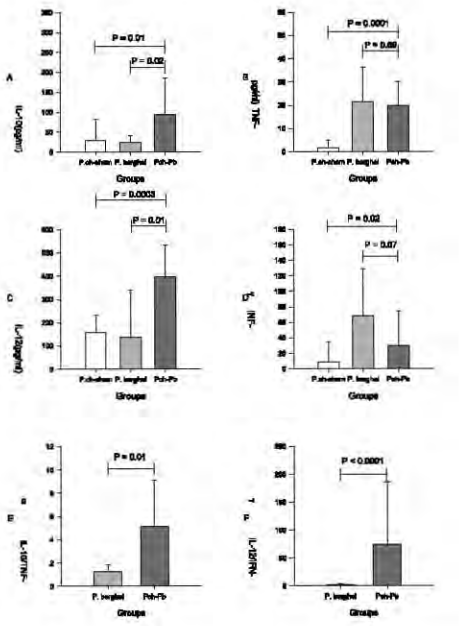
NA = not applicable

IL-12, IFN-γ, TNF-α, and IL-10 are elevated in P. chabaudi/P. berghei-*infected animals*

Plasma obtained at the time of euthanasia was used to measure the expression of ten different cytokines. There was no significant difference in expression levels of IL-1β, IL-2, IL-4, IL-5, IL-6, and GM-CSF between experimental groups (data not shown). On the other hand, the concentrations of IL-12, TNF-α, IFN-γ, and IL-10 were elevated in *P. chabaudi/P. berghei*-infected mice compared to *P. chabaudi*/sham-infected mice (Figure 19A-D). The concentrations of IL-12 and IL-10 were significantly lower in *P. berghei*-infected animals compared to *P. chabaudi/P. berghei*-infected animals, while there was no difference in the concentration of TNF-α between the two groups. *P. berghei*-infected animals had higher, although not significant, IFN-γ levels compared to *P. chabaudi/P. berghei*-infected animals. To better understand the relationship between known associated cytokines and potential disease state, we examined the ratio of IL10 to TNF-α and the ratio of IL-12 to IFN-γ. The IL-10:TNF-α and IL-12:IFN-γ ratios were higher in *P. chabaudi/P. berghei*-infected animals compared to *P. berghei*-infected animals (Figure 19E-F).

Figure 19: ***P. chabaudi/P. berghei*-infected animals have increased IL-12, TNF-α, IFN-γ, and IL-10.** Plasma samples from 3 separate experiments, obtained 9-10 days post infection at the time of euthanasia, were analyzed with a multiplex cytokine assay. (A-D) Average cytokine concentration ± SD are shown. *P. chabaudi*/sham (Pch-sham) (n=12), *P. berghei* (n=6), *P. chabaudi/P. berghei* (Pch-Pb) (n=11). (E) IL-10:TNF-α ratio of mice with detectable cytokines are shown. (F) IL-12:IFN-γ ratio of mice with detectable cytokines are shown.

Figure 19: *P. chabaudi/P. berghei*-infected animals have increased IL-12, TNF-α, IFN-γ, and IL-10

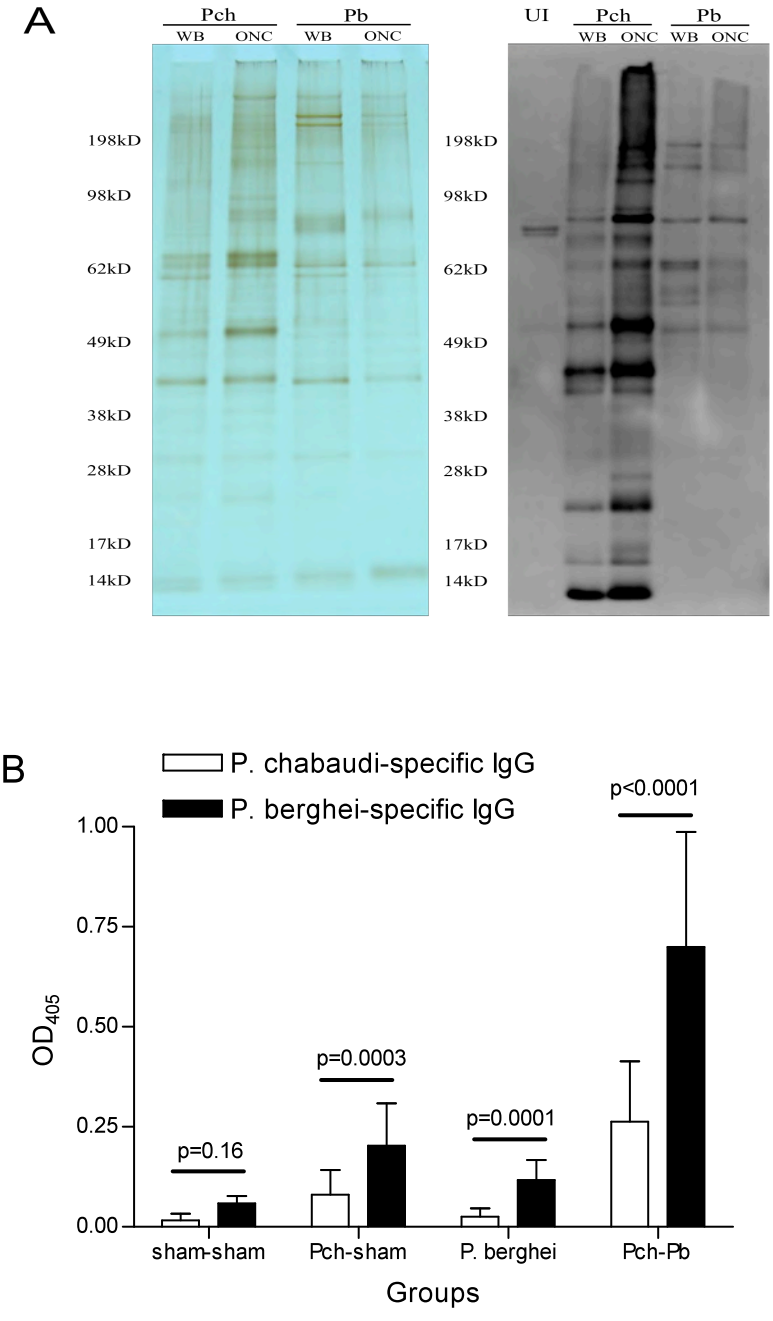


Parasite-specific and cross-reactive antibodies present in P. chabaudi/P. berghei-*infected animals*

To further characterize the model, parasite-specific total IgG antibodies were measured in sera obtained at the time of euthanasia. The presence of malaria proteins in the antigen preparations was verified by Western blot (Figure 20A). Figure 20B shows animals exposed to malaria parasites had a detectable antibody response, and furthermore, the response to *P. berghei* antigen was more robust. *P. chabaudi*/sham and *P. chabaudi/P. berghei*-infected animals had significantly higher *P. chabaudi*-specific antibody responses than sham/sham-infected animals ($P = 0.001$ and $P < 0.0003$ respectively). Detectable antibody responses to *P. berghei* antigen in *P. chabaudi*/sham-infected animals indicated the presence of cross-reactive antibodies, which is further reinforced by the robust response to *P. berghei* antigen by *P. chabaudi/P. berghei*-infected animals.

Figure 20: ***P. chabaudi/P. berghei*-infected animals have enhanced parasite-specific IgG responses.** (A) Verification of malaria antigen is shown. 0.5 μg of protein from whole blood lysate (WB) or overnight culture lysate (ONC) were loaded into each well. Protein was obtained from *P. chabaudi* (Pch) and *P. berghei* (Pb) infected animals; uninfected (UI) blood was used as a control. (Left gel) A silver stain verifying the presence of protein is shown. (Right gel) A Western blot verifying the presence of malaria specific protein is shown. Plasma from an immune mouse provided the primary Ig antibody and an HRP-labeled goat anti-mouse IgG secondary antibody was used. (B) Serum samples from 3 separate experiments, obtained at the time of euthanasia, were analyzed by ELISA and shown. IgG response to *P. chabaudi* and *P. berghei* antigen shown as average OD_{405} ± SD. Significant ($P \leq 0.02$) *P. chabaudi*-specific antibody responses were measured in *P. chabaudi*/sham and *P. chabaudi/P. berghei* animals, while significant ($P \leq 0.001$) *P. berghei*-specific antibody responses were measured in all groups except the Sham/Sham. Antibody responses to *P. berghei* are more robust in all groups except the malaria naïve mice (Sham/Sham). Sham/Sham (n=3), *P. chabaudi*/Sham (Pch-sham) (n=10), *P. berghei* (n=11), *P. chabaudi/P. berghei* (Pch-Pb) (n=11)

Figure 20: *P. chabaudi/P. berghei*-infected animals have enhanced parasite-specific IgG responses

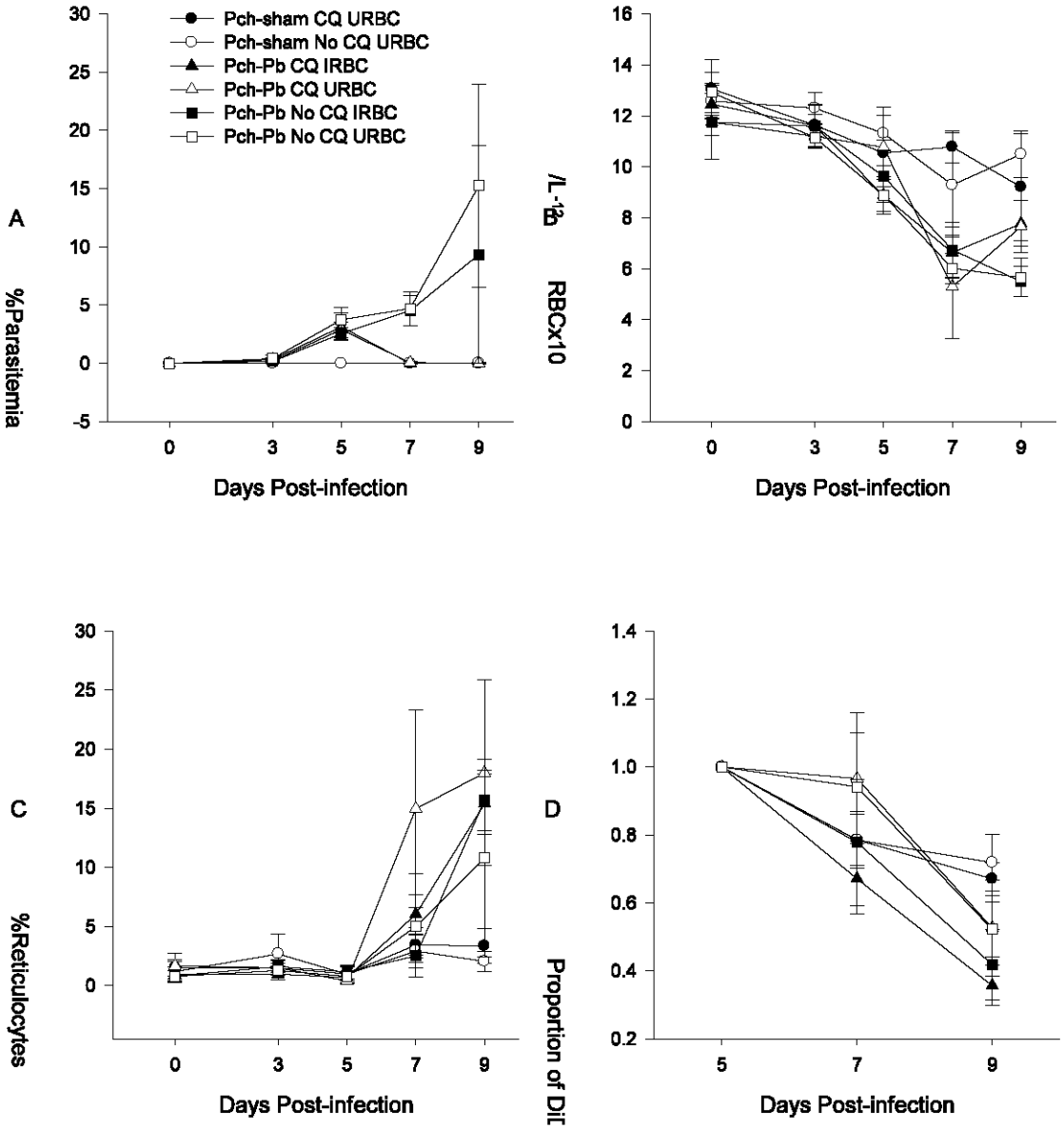


Chloroquine treatment does not prevent the development of severe anemia

To shed more light into the potential mechanisms of SMA, we treated animals with chloroquine early during infection and tracked the fate of DiD-labeled RBCs obtained from *P. chabaudi/P. berghei*-infected animals with approximately 3% parasitemia (IRBCs) or from uninfected animals (URBCs). Chloroquine treatment led to a rapid decline in parasitemia (Figure 21A) but did not prevent the development of anemia (Figure 21B). However, chloroquine-treated animals did show a faster recovery by day 9. All anemic animals had a brisk reticulocyte response by day 9 (Figure 21C). Figure 21D depicts the proportion of labeled RBCs in circulation normalized to the baseline. IRBCs injected into *P. chabaudi/P. berghei*-infected animals disappeared faster from the circulation than URBCs. Surprisingly URBCs injected into *P. chabaudi*/sham animals disappeared faster in the first two days following injection than those injected into *P. chabaudi/P. berghei*-infected animals.

Figure 21: Chloroquine treatment does not prevent development of anemia.
C57BL/6 mice were infected with 10^6 *P. berghei* following recovery from a *P. chabaudi* infection (Pch-Pb) or were sham infected with RPMI/BSA (Pch-sham). Some groups were treated with chloquine (CQ) 5 days post-infection to eliminate parasites. DiD-labeled RBCs obtained from *P. chabaudi/P. berghei*-infected animals with approximately 3% parasitemia (IRBCs) or from uninfected animals (URBCs) were transferred into Pch-sham and Pch-Pb animals at 5 days post-infection and tracked by flow cytometry. Average parasitemia (A), RBC counts (B), and reticulocyte levels (C) were monitored for 9 days. (D) The proportion of labeled RBCs in circulation normalized to baseline values is shown.

Figure 21: Chloroqine treatment does not prevent development of anemia



Discussion

We aimed to develop and characterize a relevant murine model of SMA that is highly reproducible. Infection of C57BL/6 mice with *Plasmodium berghei* ANKA is uniformly fatal whereas infection with *P. chabaudi* AS leads to severe anemia with high parasitemia followed by full recovery[115;117]. We determined that infection with *P. chabaudi* AS followed after recovery by infection with *P. berghei* ANKA results in anemia with relatively low level parasitemia in a highly reproducible manner. Major advantages of this model over a recently published rodent model[121] are the occurrence of synchronized infections, the predictable timing of the anemia, and a short preparative time of only 6-8 weeks. Another important advantage of our model is the strain of mouse that we use. C57BL/6 is the most commonly used background for genetically modified mice allowing for the exploration of the role of different genes in the pathogenesis of SMA.

Our model has a number of features that are very relevant to human *P. falciparum* infections. SMA is more often found in areas of endemic malaria transmission[8] where children suffer repeated infections[134]. This observation suggests that some level of immunity or at least immune stimulation is required for SMA to occur. Thus, our model is in agreement with this observation. Another feature of this model is that, as in humans, the level of anemia is out of proportion to the parasite burden[22;133;141].

The carefully orchestrated balance between pro-inflammatory and regulatory cytokines produced by the host is important in determining the outcome of malaria infection[142;143]. Therefore, we measured pro-inflammatory and anti-inflammatory cytokines. We found that *P. berghei*-infected animals produced higher levels of IFN-γ

and TNF-α relative to their respective regulatory cytokines IL-12 and IL-10. This unregulated production of IFN-γ and TNF-α seems central to the development of CM[144;145]. Normally, IL-12, produced by macrophages and dendritic cells[146;147], induces the production of IFN-γ by NK cells and CD8+ T cells. However, in *P. berghei*-infected mice IFN-γ is produced in an IL-12-independent manner[148], which explains the high IFN-γ/IL-12 ratios in these animals. IL-12 has been shown to play an important role in controlling parasitemia[120;149-154] which may explain the relatively low parasitemias in *P. chabaudi/P. berghei*-infected animals. IL-12 also plays a role in the stimulation of erythropoiesis[29;155] which may account for the excessive extramedullary hematopoiesis and brisk erythropoietic response in *P. chabaudi/P. berghei*-infected animals. The other important regulatory cytokine is IL-10, which normally counters the action of TNF-α and IL-12 by downregulating their production[156-158]. *P. berghei*-infected animals produce inappropriately low levels of this cytokine. Thus, sequential *P. chabaudi-P. berghei* infection restored the normal regulatory relationship between IL-12, IL-10, IFN-γ, and TNF-α. These data suggest that the *P. chabaudi/P. berghei* infection model can be an important tool to study the role of these cytokines in the pathogenesis of CM and SMA. In addition, the model can serve to study how innate immune responses change with repeated infections, an important but poorly understood phenomenon.

P. berghei infection of *P. chabaudi*-experienced mice elicited strong cross-reactive antibody responses against *P. chabaudi* antigens as well as against *P. berghei*. Surprisingly, *P. chabaudi*/sham-infected mice showed a higher antibody response against *P. berghei* antigens than against antigens from the homologous parasite. The increased antibody responses in *P. chabaudi/P. berghei*-infected animals may be in part due to their

ability to produce IL-12 which has been found to be critical for production of cytotoxic antibodies[159]. The role of these antibodies in the development of SMA is not clear. Jarra *et al*.[160] demonstrated that although there was cross-reactive antibody recognition of *P. berghei* by anti-*P. chabaudi* serum and vice-versa, immune serum had little effect on the rise of parasitemia of homologous parasites and no effect on heterologous parasitemia. Possible mechanisms of antibody-mediated uninfected red cell destruction include opsonization of erythrocytes by antibodies that recognize self antigens, or by complement activated by immune complexes, both leading to erythrophagocytosis.

In an attempt to further understand the fate of RBCs we performed experiments in which we treated mice with chloroquine early during the infection and infused labeled RBCs by retro-orbital injection to track their disappearance from the circulation. Chloroquine treatment did not prevent the development of anemia. These data suggest that the mechanism of anemia in our model is set in motion early on during the infection. Because the percent of infused RBCs in the peripheral circulation kept declining from the time of infusion, we can conclude that these RBCs were removed at a rate faster than native unlabeled RBCs and thus, to some extent, all the infused RBCs were probably damaged. However, infused RBCs from naïve uninfected animals had longer lifespan than RBCs from *P. chabaudi/P. berghei*-infected animals, suggesting that the latter had surface changes that may predispose them to destruction or that infusion of additional IRBCs, although small as a percentage, increases the trapping of all RBCs. Paradoxically, the survival of RBCs from uninfected animals was lower during the first two days post injection in *P. chabaudi*/sham-infected mice than in *P. chabaudi/P. berghei*-infeced mice. These results are remarkably consistent with published animal and human studies

showing accelerated removal of heat-treated RBCs during *P. falciparum* infection in patients with splenomegaly[125], premature removal of uninfected RBCs in mice[124], and destruction of RBCs despite appropriate treatment of malaria infection in humans[23;126;161].

The exact mechanism of SMA in our model remains to be elucidated. One possible mechanism is erythrophagocytosis and/or cytoadherance of RBCs to macrophages. This is supported by the increased levels of phagocytosis in the liver, as well as the increased susceptibility to phagocytosis *in vitro*. Erythrophagocytosis has also been reported in the spleens and livers of *P. chabaudi*-infected B6 and A/J mice[37] and is also a common finding in the spleens and livers of patients who die of *P. falciparum* malaria[162;163]. RBCs of patients with SMA also have increased susceptibility to erythrophagocytosis *in vitro*[35]. Unfortunately, our attempts to test the role of macrophages by eliminating them with clodronate proved too toxic. Macrophages seemed resistant to clodronate after *P. chabaudi* infection and, therefore, two rounds of treatment were required, one before *P. chabaudi* infection and one before *P. berghei* infection. Treatment with cobra venom factor resulted in decreased erythrophagocytosis without an effect on the anemia, suggesting that the erythrophagocytosis was complement-mediated but played no significant role in the development of anemia. An alternative explanation is that RBCs are being sequestered in the liver and/or spleen. In the spleen, in particular, blood can flow directly from the arterioles into the red pulp sinusoids (closed circulation) or into the cordal meshwork (open circulation) which can lead to entrapment of infected and uninfected red cells[164-167]. However, more recent studies have not shown a straightforward relationship between the pattern of blood flow and the level of parasitemia[168;169]. Nonetheless, we speculate whether following a *P.*

chabaudi infection the spleen retains an open circulation that allows control of parasitemia during a subsequent infection but at the same time results in entrapment of uninfected red cells leading to SMA.

In summary, we have presented a practical and novel mouse model of severe malarial anemia that has features that are relevant to *P. falciparum* infection in humans. As in humans, pre-immunity is a key feature of the model. SMA develops in the presence of relatively low parasitemia following *P. berghei* infection of animals whose immune system was previously stimulated by *P. chabaudi*-infection. The immune responses are characterized by relatively high IL-12 and IL-10 resulting in high IL-10/TNF-α and IL-12/IFN-γ ratios compared to *P. berghei*-infected mice as well as strong antibody responses which are higher against *P. berghei* than against *P. chabaudi*. This model provides an excellent platform to study the role protective immune responses against CM and SMA in the pathogenesis of these conditions, changes in the innate response to malaria with repeated infections, and the fate of uninfected RBC during SMA.

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Chapter 6

General Discussion/Conclusions

Malaria is a tropical parasitic disease responsible for ~1 million deaths annually[3], the majority from complications such as severe malarial anemia (SMA), yet the pathogenesis of severe anemia is not understood. Additionally, it is known that there is greater destruction of red cells than can be accounted for by the parasite alone, but the question of what is happening to the uninfected red cells remains unanswered. The goal of this project was to investigate the pathogenesis of SMA by studying the fate of uninfected red cells in two different mouse models, with an emphasis on the role of complement and complement regulatory proteins.

The first model we utilized was a well established model of malarial anemia involving the rodent parasite *P. chabaudi* in C57BL/6 mice. The availability of Crry heterozygote and C3 deficient animals on this genetic background allowed for the investigation of these components in the pathogenesis of SMA. We ascertained that Crry partial deficiency did not result in more severe anemia evident by blood counts, but did result in a higher level of erythrophagocytosis which could be negated with supplementation of the deficient protein. These findings indicate that Crry is important in red cell protection during a malaria infection.

Crry functions to protect host cells from complement attack[108;109]. If Crry is important in red cell protection during a malaria infection, does that necessarily mean C3 is deleterious to the red cells and/or causes more severe anemia? Our results in C3 deficient animals suggest that it may be harmful, but perhaps not to as great of a degree

as we would expect if C3 were directly responsible for the pathogenesis of SMA. C3 deficient animals did have less severe anemia; however, these animals still developed anemia and we saw clearance and sequestration of transferred red cells.

Unfortunately, studying the uninfected cell population in the *P. chabaudi* model is complicated by a high level of parasitemia in infected animals. We observed parasitemia levels as high as 40%, which means 40% of the red cell destruction was due to direct lysis. A problem that exists is the lack of a mouse model with a low parasite burden, development of anemia, and reproducibility. For those reasons, we developed the second model we utilized to investigate SMA. To be able to compare our results from the first model and be able to use the same genetically modified animals, we developed our model in C57BL/6 mice. *P. berghei* infection following recovery from *P. chabaudi* infection in C57BL/6 animals resulted in anemia with a low level parasitemia. Characterization of the model revealed similarities to anemia in *P. chabaudi* infected animals, such as increased organ pathology and erythrophagocytosis of infected and uninfected red cells. This model can now be used to evaluate factors such as complement regulatory proteins and complement in the development of anemia.

Discussion and questions stemming from Crry and C3 studies

Our work on complement regulatory proteins surprisingly showed no clear association between Crry and anemia in regards to hematocrit. However, using erythrophagocytosis as a surrogate marker for anemia revealed a role for Crry in protecting red cells. Why did we not see more severe anemia at the blood level if Crry is important in red cell protection during a malaria infection? The most likely answer is

because while more red cells were being destroyed in animals with the Crry deficiency they were able to compensate for the red cell loss by extramedullary hematopoiesis, which is evident in the liver and spleen. Another possibility is that partial deficiency in Crry may not confer enough of a deleterious effect to equate to more severe anemia evident in the RBC count. I do not think that is actually the case, but it is interesting to speculate whether there is a certain "cutoff" level where Crry is protective enough to negate deleterious events seen in the blood. It would be interesting to evaluate the development of SMA in a mouse completely deficient in Crry; however, since Crry knockout mice have an impaired C3 activity compared to wild-type animals, it is not possible to perform that comparison.

However, Crry is not the only complement regulatory protein present on the surface of mouse red cells. Since Crry has overlapping function with CD55, which has also been shown to be decreased in children with SMA, perhaps CD55 also has a role in protecting mouse red cells during a malaria infection. There are CD55 knockout animals that could be used in studies similar to those performed in chapter 3. This was an avenue we had initially wanted to investigate, but unsuccessful breeding led us to focus our efforts on Crry. Additionally, we consistently observed that our CD55 knockout animals suffered from severe ulcerative dermatitis and we were not sure how that would interfere/effect the malaria infection. However, studies with CD55 deficient animals may reveal a role for the regulator in red cell protection, which could work in concert with Crry.

A fundamental question that remains from the Crry studies is, what mechanism is causing the development of anemia? Our data certainly implicate erythrophagocytosis in

the liver. Erythrophagocytosis is probably also occurring in the spleen, but because of the massive cellular expansion we are not able to clearly evaluate this possibility with our methods. Deficiency in Crry is clearly resulting in increased erythrophagocytosis *in vivo* and enhanced phagocytosis *in vitro*. Why is that happening? Does it have to do with increased C3 deposition on the surface of the red cell or are there other host or parasite factors causing enhanced clearance of these particular cells. Given the *in vitro* data, I tend to believe there is modification to the red cells, which is most likely deposition of C3b and/or binding of immune complexes. Unfortunately, all attempts to measure C3b on the surface of red cells were unsuccessful. It would be interesting, however, to find a method that successfully measures C3 on the surface of these mouse red cells. Another approach would be to stain for C3 in tissue sections via immunohistochemistry and look for colocalization with red cells and macrophages. We have attempted C3 immunohistochemical staining, but have had only limited success. Besides C3b and immune complexes, it is possible that parasite proteins are being deposited on the surface of uninfected red cells. Some studies have shown rhoptry proteins on uninfected cells[39;132], presumably deposited during aborted merozoite invasion. It is also possible that other merozoite surface proteins released during schizont rupture could be attaching to neighboring uninfected red cells. These foreign particles on the host red cells could effectively be marking that cell as "not host" and subsequently those cells would be targeted for removal, likely by antibody-mediated mechanisms. Alterations to the red cell can also affect the integrity of the red cell membrane, which could result in mechanical clearance in organs such as the spleen and liver.

Our results showing a protective role for Crry logically led to a question regarding the role of C3; however, we have not fully explored the role C3 has in the pathogenesis of SMA. Experiments that looked only at blood parameters showed C3 knockout animals had less severe anemia, which suggests C3 is harmful to red cells during a malaria infection. These experiments need to be repeated with other parameters monitored, much as was done for the experiments in chapter 3. If, as our data suggests, erythrophagocytosis is a mechanism involved in the development of SMA, I would suspect we would see significant declines in the level of erythrophagocytosis observed in C3 knockout animals. Also, studies with supplementation of C3 into deficient animals should be conducted to see if addition of C3 restores the increased severity of anemia.

Examining Crry and C3 separately begins to elucidate the roles of each component; however, complement and complement regulatory proteins are inherently connected because of their biological functions. Therefore, what interactions do Crry and C3 have, if any, in the context of malaria and the development of SMA? Are these interactions important to the pathogenesis? Crry has been shown to be extremely important in protecting mouse red cells from complement[110]; therefore it makes sense that in the context of an infection where complement is activated there would be an interaction between the regulatory proteins and complement. Additionally, data from children with SMA showing increased C3b deposition on red cells that have deficiencies in complement regulatory proteins[32] suggests there is an interaction. We started addressing the question of interactions between Crry and C3 with the transfer experiment discussed in chapter 4. The increased clearance of transferred Crry deficient red cells was decreased when animals lacked C3, suggesting C3 in combination with Crry

deficiency can lead to more severe disease. While transfer of red cells provides a means to evaluate clearance of different red cell types in the presence and absence of C3, it has limitations in the conclusions we can draw. However, transfer of red cells completely deficient in Crry is the only way we can really evaluate interactions of those cells in a wild-type setting since Crry knockout animals have less complement activation potential than our other animals. Another way we could examine the role of Crry and C3 together is by using double knockout animals; i.e. they lack both Crry and C3. I would expect to see less severe disease in these animals compared to $Crry^{+/-}$ animals.

These studies confirmed a role for complement and complement regulatory proteins in a malaria infection in mice. The exact role for each of these components still needs to be fully elucidated, but the evidence suggests red cells are protected from phagocytosis by the regulatory protein Crry, which when deficient predisposes red cells to clearance by an unclear mechanism. This could be complement-mediated or it may be mediated by means independent of complement. However, complement appears to have a role in exacerbating the severity of disease. A better understanding of the contributions that complement and complement regulatory proteins have in the pathogenesis of SMA will be garnered when these aspects are examined in a model that is more representative of human infection.

Discussion and questions stemming from new anemia model

The purpose of this portion of my project was to develop a new model of malarial anemia that was representative of *P. falciparum* infection and do the initial characterization. We successfully developed the model and characterized the basic

pathology and immune response. The main question we have from this work is what is the major mechanism of anemia in this model? Early evidence indicates erythrophagocytosis is involved and our *in vitro* phagocytosis assays show increased susceptibility to phagocytosis of RBCs from model animals. To evaluate this mechanism further, the role of macrophages needs to be clarified. One means of doing this is to delete macrophages from the animals and then evaluate the malaria infection. We tried using clodronate to do this, but were unsuccessful in fully clearing spleen and liver macrophages and the clodronate was extremely toxic in our animals. One possibility is to change the method of clodronate administration, perhaps by using IV treatment, which is supposed to selectively deplete macrophages in the spleen and liver, instead of IP, which depletes all macrophages, and see if there is less toxicity. Another possibility is to use antibody depletion to try and specifically target liver macrophages. However, I suspect elimination of liver and spleen macrophages could complicate matters because there would likely be uncontrolled parasite growth due to lack of phagocytic clearance of infected cells.

Another way to evaluate the mechanism of RBC clearance/destruction is to track labeled red cells. Our initial RBC transfer experiments indicate red cells are trafficked to the liver and spleen. One issue we observed is that even in uninfected animals, labeled cells were being cleared more rapidly than host red cells indicating there was some damage to the transferred cells. Our lab is now examining the use of *in vivo* labeling in an attempt to avoid any damage from the *ex vivo* labeling process. We can make use of *in vivo* imaging technology to monitor the labeled red cells at multiple timepoints, which should allow for correlations between clearance from the peripheral blood and

appearance in tissues. Additionally, we can harvest organs that are shown to have fluorescent activity by IVIS and examine the histology by fluorescent microscopy. That should provide an answer as to what is happening to the red cells; i.e. are they being phagocytosed or are they being trapped by other means?

Another question stemming from this work is, what will studies with our genetically modified animals reveal? The development of this model allows us to investigate our previous questions regarding the role of complement and complement regulatory proteins in a system where high parasite burdens are no longer an issue. However, do complement regulatory proteins have any role in this model? Flow cytometric analysis of model animal red cells revealed a decline in Crry expression at the final time-point compared to baseline values. These data correspond with what we saw in *P. chabaudi* infected mice, which provides evidence that Crry could have a role in protection of red cells in the new model as well. The next step is to perform the same kind of experiments as discussed in chapters 3 and 4 using this new model. In addition, experiments in CD55 knockout animals can be conducted to ascertain whether CD55 is important in red cell protection during a malaria infection. Also, use of double knockout animals, such as $Crry^{-/-}/C3^{-/-}$ animals, can address the interplay between complement regulatory proteins and complement. It will be of particular interest to see if differences in the level of anemia are observed in this new model.

Conclusions

Severe malarial anemia is complex and there is no one answer to its pathogenesis. Animal models provide a good foundation for evaluating host-parasite interactions that

could be involved in the development of anemia. Data we have obtained and data from in patients with SMA clearly indicate a role for complement regulatory proteins in the protection of red cells during a malaria infection. Complement also contributes to the development of SMA, but if that contribution is direct or indirect still needs to be ascertained. Understanding the interplay between complement, complement regulatory proteins, and other components of the immune response is going to be crucial to understanding the pathogenesis of SMA and being able to develop effective treatment strategies. However, while the information garnered in this project, as well as the creation of a new animal model helps us understand the role that complement plays in SMA, we are still a long way away from implementing a reasonable and effective treatment and/or prevention for patients that suffer from SMA.

Chapter 7

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